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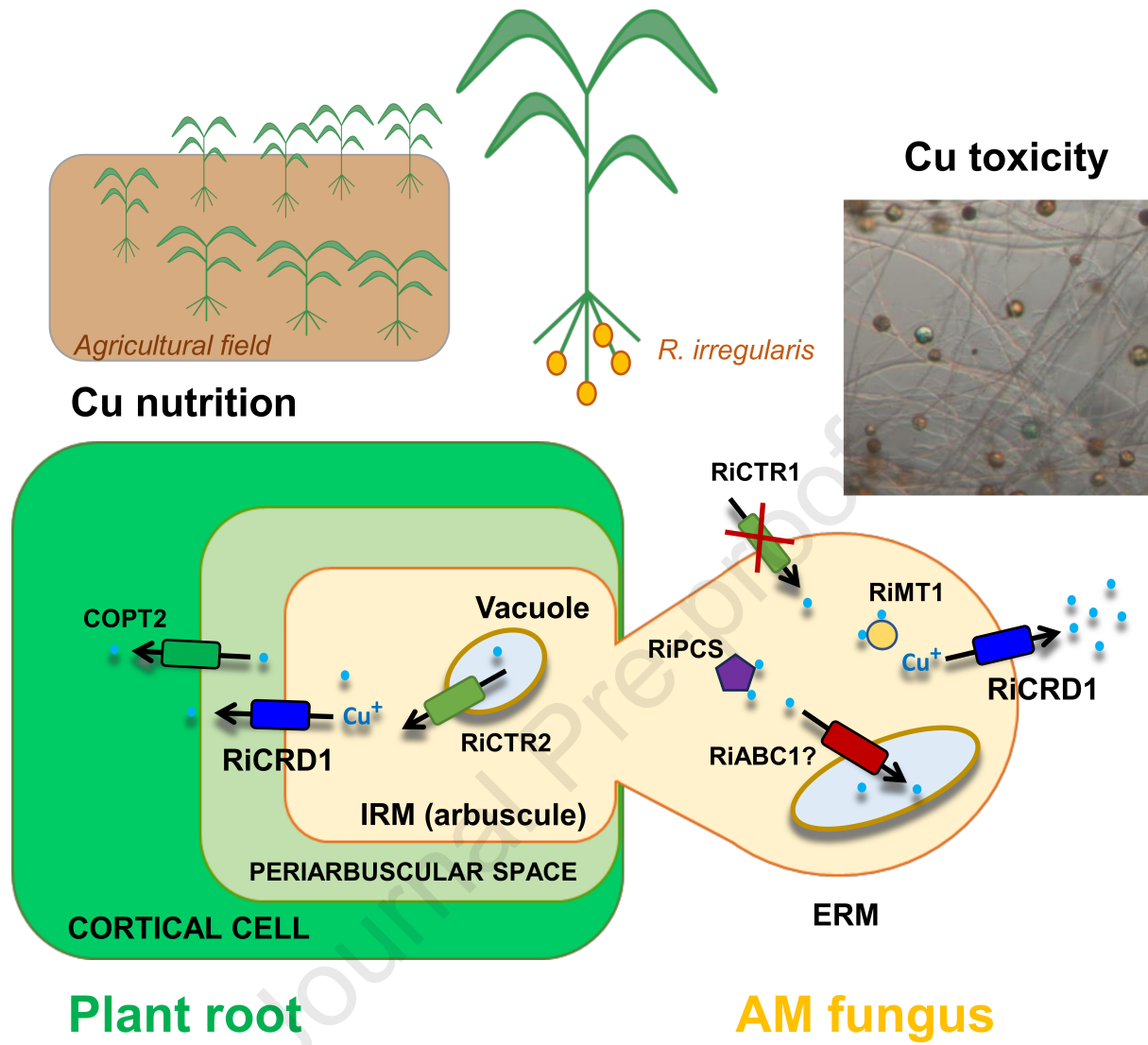
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The arbuscular mycorrhizal fungus *Rhizophagus irregularis* uses the copper exporting ATPase RiCRD1 as a major strategy for copper detoxification

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1 Abstract

2 Arbuscular mycorrhizal (AM) fungi establish a mutualistic symbiosis with most land
3 plants. AM fungi regulate plant copper (Cu) acquisition both in Cu deficient and
4 polluted soils. Here, we report characterization of *RiCRD1*, a *Rhizophagus irregularis*
5 gene putatively encoding a Cu transporting ATPase. Based on its sequence analysis,
6 *RiCRD1* was identified as a plasma membrane Cu⁺ efflux protein of the P_{1B1}-ATPase
7 subfamily. As revealed by heterologous complementation assays in yeast, *RiCRD1*
8 encodes a functional protein capable of conferring increased tolerance against Cu. In the
9 extraradical mycelium, *RiCRD1* expression was highly up-regulated in response to high
10 concentrations of Cu in the medium. Comparison of the expression patterns of different
11 players of metal tolerance in *R. irregularis* under high Cu levels suggests that this
12 fungus could mainly use a metal efflux based-strategy to cope with Cu toxicity.
13 *RiCRD1* was also expressed in the intraradical fungal structures and, more specifically,
14 in the arbuscules, which suggests a role for *RiCRD1* in Cu release from the fungus to
15 the symbiotic interface. Overall, our results show that *RiCRD1* encodes a protein which
16 could have a pivotal dual role in Cu homeostasis in *R. irregularis*, playing a role in Cu
17 detoxification in the extraradical mycelium and in Cu transfer to the apoplast of the
18 symbiotic interface in the arbuscules.

19

20 **Keywords:** arbuscular mycorrhiza; copper homeostasis; *Rhizophagus irregularis*; heavy
21 metal ATPase; metallothionein; phytochelatin synthase

22 **Abbreviations:** AM, arbuscular mycorrhiza; Cu, copper; ERM, extraradical mycelium;
23 HMA, heavy metal ATPase; IRM, intraradical mycelium; PC, phytochelatin; PCS,
24 phytochelatin synthase; ROS, reactive oxygen species

25

26

27 **1. Introduction**

28 Copper (Cu) homeostasis is tightly controlled in all organisms due to the dual
29 effect of this transition metal. Cu is an essential micronutrient, but it is a toxic element
30 when in excess. It is actively used as a cofactor by cytochrome *c* oxidases, superoxide
31 dismutases and multicopper oxidases, among other enzymes that are involved in
32 important processes such as respiration, reactive oxygen species (ROS) removal and Fe
33 nutrition (Festa and Thiele, 2011; Linder, 1991). The key role of Cu in metabolic
34 processes is associated with its ability to switch between an oxidized (Cu^{2+}) and a
35 reduced (Cu^+) state, resulting in the acceptance and donation of single electrons in
36 cellular redox reactions. However, these redox properties also make this metal toxic
37 when present at high concentrations. Cu excess can damage DNA, proteins and lipids
38 through the generation of ROS by Fenton like reactions. It can also displace other metal
39 cofactors such as iron and zinc (Halliwell and Gutteridge, 1984; Macomber and Imlay,
40 2009).

41 Although Cu is a trace element, Cu toxicity has become an agricultural and
42 environmental problem for decades owing mainly to anthropogenic activities. High Cu
43 concentrations are toxic to soil inhabitants. However, some soil microorganisms have
44 developed adaptative mechanisms that allow them to survive and grow in environments
45 with high Cu concentrations (Bååth, 1989; Ferrol *et al.*, 2009). Arbuscular mycorrhizal
46 (AM) fungi, obligate biotrophs of higher plants, constitute one of the most prominent
47 groups of soil microorganisms (Pozo *et al.*, 2021; Shi *et al.*, 2023). AM fungi belong to
48 the subphylum Glomeromycotina within the phylum Mucoromycota and establish a
49 widespread mutualistic symbiosis with most land plants (Brundrett and Tedersoo, 2018;
50 Spatafora *et al.*, 2016). The fungus biotrophically colonizes the root cortex and develops

51 specialized structures, the arbuscules, to facilitate nutrient exchanges between
52 symbionts (Luginbuehl and Oldroyd, 2017). Simultaneously, the fungus develops an
53 extensive network of extraradical hyphae that can absorb nutrients beyond the depletion
54 zone that develops around the roots, providing a new pathway, the mycorrhizal
55 pathway, for the uptake of low mobility macronutrients, such as phosphorus, and
56 micronutrients (Cu, Zn) in soil (Coccina et al., 2019; Lanfranco *et al.*, 2018; Moreno
57 Jiménez *et al.*, 2023; Wipf *et al.*, 2019). In return, the AM fungus receives up to 20 %
58 of the photosynthetically fixed carbon from the plant in the form of lipids and sugars
59 (An *et al.*, 2019; Brands and Dörman 2022; Jiang *et al.*, 2017; Roth and Paszkowski,
60 2017). Mechanisms of phosphorus and nitrogen transport through the mycorrhizal
61 pathway have been widely studied (Ferrol *et al.*, 2019; Hui *et al.*, 2022; Wang *et al.*,
62 2020; Xie *et al.*, 2022), but little is known about the components involved in
63 micronutrient nutrition in AM associations (Ferrol *et al.*, 2016; Ruytinx *et al.*, 2020). As
64 genetic manipulation of AM fungi remains challenging, the main advances have been
65 performed on the host plant. In recent years functional analysis of AM fungal genes
66 highly expressed in the intraradical mycelium has been achieved by using host-induced
67 and virus-induced gene silencing strategies (Ezawa *et al.*, 2020; Helber *et al.*, 2011;
68 Wang *et al.*, 2023). However, more studies are required to improve the applicability of
69 these methodologies since their efficiency is unpredictable and gene and construct
70 dependent (Hartmann *et al.*, 2020).

71 In soils with low Cu levels, the contribution of the mycorrhizal pathway to plant
72 Cu nutrition can be up to 75% (Lee and George, 2005; Li *et al.*, 1991). To our
73 knowledge only two components of the mycorrhizal Cu uptake have been described so
74 far: RiCTR1, a *Rhizophagus irregularis* plasma membrane Cu transporter of the CTR
75 family whose expression in the extraradical mycelium (ERM) increases under Cu

76 deficiency but decreases under Cu toxicity (Gómez-Gallego *et al.*, 2019), and
77 *MtCOPT2*, a *Medicago truncatula* plasma membrane Cu transporter specifically
78 expressed in arbuscule-colonized cortical root cells (Senovilla *et al.*, 2020). However, it
79 is currently unknown how Cu is released by the fungus to the apoplast of the symbiotic
80 interface developed in the cortical cells colonized by arbuscules.

81 Under conditions of supraoptimal levels of Cu, AM fungi are able to alleviate
82 metal toxicity in the plant. Different mechanisms have been proposed to explain the
83 protective effect of the AM symbiosis under heavy metal stress (Ferrol *et al.*, 2016;
84 Gómez-Gallego *et al.* 2022; Shi *et al.* 2019). One of the mechanisms to mitigate the
85 effect of Cu toxicity is the reduction of the effective concentration of metal available to
86 the plant through immobilization of the metal in the intraradical and extraradical
87 structures of the fungus (Cornejo *et al.*, 2013; González-Guerrero *et al.*, 2008). This is
88 possible thanks to the existence in the fungus of a complex regulatory system that
89 controls Cu homeostasis and avoids Cu stress in the cytosol. This system includes metal
90 binding to the cell wall, reduction of metal uptake, intracellular buffering through the
91 activity of intracellular chelators, such as metallothioneins and glutathione, and
92 compartmentalization of Cu in vacuoles or spores (Ferrol *et al.*, 2009; Ma *et al.*, 2022).
93 However, a mechanism related to the control of Cu efflux has not been described yet.

94 Export of metal ions, such as Cu, Zn and Cd, usually takes place through P_{1B}-
95 type ATPases, commonly known as HMAs (Heavy Metal ATPases). These proteins
96 couple ATP hydrolysis to the transport of a heavy metal across cellular membranes in a
97 multistep process, which includes the specific recognition of the metal (Palmgren and
98 Nissen, 2011; Salustros *et al.* 2022). They possess six or eight transmembrane domains,
99 a conserved intramembranous CPX signature needed for metal translocation, and
100 cytoplasmic metal binding domains, which makes them different to their archetypal P-

101 ATPases counterparts (Arguello *et al.*, 2007; Solioz and Vulpe, 1996). The genome of
102 the model fungus *Rhizophagus irregularis* has four candidate genes putatively encoding
103 P_{1B}-type ATPases (Tamayo *et al.*, 2014). *RiCCC2.1*, *RiCCC2.2* and *RiCCC2.3* are
104 orthologs of the *Saccharomyces cerevisiae* *CCC2*, which encodes a Cu-ATPase
105 transporting Cu to Cu containing proteins in the trans-Golgi region (Yuan *et al.*, 1995).
106 *RiCRD1* is ortholog of *CaCRD1* of the pathogenic yeast *Candida albicans*, which
107 encodes a P_{1B}-ATPase that exports excess Cu out of the cell, providing Cu resistance
108 (Riggle and Kumamoto, 2000; Weissman *et al.*, 2000).

109 The aim of this work was to characterize the *R. irregularis* *RiCRD1* gene to
110 better understand the mechanisms of metal homeostasis in AM fungi. Our data suggest
111 that the *RiCRD1* gene product plays a role in *R. irregularis* metal tolerance by
112 detoxifying metal excess out of the fungus as well as in symbiotic Cu transport by
113 releasing Cu from the arbuscules to the apoplast of the symbiotic interface. Our gene
114 expression data also indicate that *R. irregularis* mainly uses a metal efflux based-
115 strategy to cope with Cu toxicity.

116

117 2. Materials and methods

118 2.1. Biological materials and growth conditions

119 *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A.
120 Schüßler DAOM 197198 monoxenic cultures were established on Ri T-DNA
121 transformed carrot (*Daucus carota* L. clone DC2) roots in two-compartment Petri
122 dishes filled with solid M medium (Chabot *et al.*, 1992), according to St-Arnaud *et al.*
123 (1996) with some modifications. Briefly, cultures were started in one compartment of
124 the Petri dish by placing some non-mycorrhizal carrot root fragments together with a

125 fungal inoculum containing ERM, mycorrhizal roots and spores. Plates were incubated
126 in the dark at 24°C for 6-8 weeks until the other compartment was densely colonized by
127 the fungus and roots. The oldest compartment was removed and filled with liquid M
128 medium without sucrose (M-C medium) and the fungal mycelium was allowed to
129 colonize this compartment (hyphal compartment) during the two subsequent weeks
130 (Control plates) (Fig. 1).

131 For the Cu deficiency treatment, monoxenic cultures were started with roots and
132 an AM fungal inoculum previously grown in M media without Cu and established in M
133 media without Cu. For treatments with high Cu or Cd concentrations, the M-C medium
134 of the hyphal compartment was removed and replaced with fresh liquid M-C medium
135 (Control, 0.5 μM CuSO_4) or with M-C medium supplemented with 250 μM CuSO_4 , 500
136 μM CuSO_4 or 45 μM CdSO_4 . The time of medium exchange was referred as time 0.
137 Mycelia were collected 1, 2 and 7 days after Cu addition and 1, 3, 6, 12, 24 and 48
138 hours after Cd supplementation. ERM of all treatments was frozen in liquid N and
139 stored at - 80°C until used.

140 For gene expression comparison between ERM and IRM (intraradical
141 mycelium), several non-mycorrhizal carrot roots pieces were placed on the top of a
142 densely fungal colonized compartment and grown for 15 days at 24°C. Roots were
143 carefully collected with tweezers under a binocular microscope trying to remove the
144 attached extraradical hyphae, and frozen in liquid N and stored at - 80°C until used. An
145 aliquot of root fragments was separated to estimate mycorrhizal colonization.

146 *R. irregularis* ERM was also collected from mycorrhizal plants grown in the *in*
147 *vivo* whole plant bidimensional experimental system described by Pepe *et al.* (2017)
148 with some modifications. Briefly, chicory (*Cichorium intybus* L.) seeds were surface-
149 sterilized and germinated for 10-15 days in sterilized sand. Seedlings were transplanted

150 into 50 mL pots filled with sterilized sand and inoculated with an inoculum obtained
151 from monoxenic cultures. Pots were placed in sun-transparent bags (Sigma-Aldrich,
152 B7026) and maintained during one month in a growth chamber at 24 / 21°C day/night
153 and 16 h light photoperiod. The root system of each plant was cleaned, wrapped in a
154 nylon net (41 μM mesh, Millipore NY4100010) and placed between two 13 cm
155 diameter membranes of mixed cellulose esters (0.45 μm pore diameter size, MF-
156 Millipore HAWP14250) in 14 cm diameter Petri dishes having a hole on the edge to
157 allow plant shoot growth and containing sterilized sand (Fig. 1). Petri plates containing
158 plants were sealed with parafilm, wrapped with aluminum foil, placed into sun-
159 transparent bags and maintained in a growth chamber. Plants were watered weekly with
160 a 0.5 \times modified Hoagland nutrient solution containing 125 μM KH_2PO_4 and 0.16 μM
161 CuSO_4 (control treatment) or without Cu (Cu deficiency treatment). Petri dishes were
162 opened 2 weeks after preparing the root sandwiches, and ERM spreading from the
163 nylon net onto the membranes was collected with tweezers, frozen in liquid N and
164 stored at - 80°C until used. Roots wrapped in the nylon net were also frozen and stored
165 at - 80°C. An aliquot of the roots was separated to estimate mycorrhizal colonization.

166 Tomato (*Solanum lycopersicum* L., cv. Moneymaker) mycorrhizal roots were
167 collected from plants grown in pot cultures. Briefly, germinated seeds were transferred
168 to 1.5 L pots containing a sterile mixture of sand:vermiculite (1:1, v/v) supplemented
169 (10 %) with a substrate-based inoculum of *R.irregularis*. Plants were grown in a
170 controlled environmental chamber with 65-75% relative humidity, day/ night
171 temperatures of 25/ 18°C, and a photoperiod of 16 h at 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Roots
172 were harvested 8 weeks after inoculation.

173 The *Saccharomyces cerevisiae* strains used in this study were the mutants
174 DTY113 (*cup1 Δ*) and WYT (*yap1 Δ*), lacking the metallothionein CUP1 and the

175 transcription factor *yap1*, respectively (Tamai *et al.*, 1993; Kuge and Jones, 1994).
176 Detailed characteristics of yeast strains are listed in Table S1. Yeast cells were
177 maintained on YPD or minimal synthetic dextrose (SD) medium, supplemented with the
178 appropriate amino acids.

179 2.2. Mycorrhizal colonization

180 Mycorrhizal colonization was assessed after trypan blue staining (Phillips and
181 Hayman, 1970) according to the Trouvelot method (Trouvelot *et al.* 1986). The
182 abundance of AM fungus in the roots was also determined molecularly by determining
183 the expression levels of the *R. irregularis* elongation factor 1 α (*RiEF1 α* ; GenBank
184 Accession No. DQ282611), using as internal control the elongation factor 1 α of the
185 corresponding host plant (*D. carota* *DcEF1 α* , GenBank Accession No.
186 XM_017391845; *C. intybus* *CiEF1 α* , GenBank Accession No. KP752079).

187 2.3. RNA isolation and cDNA synthesis

188 The Plant RNeasy Kit (Qiagen) was used to extract total RNA from the ERM and
189 mycorrhizal carrot roots developed in monoxenic cultures following manufacturer's
190 instructions. Total RNA from mycorrhizal chicory roots was extracted using the
191 phenol/SDS method followed by LiCl precipitation as described by Kay *et al.* (1987).
192 The isolated RNAs were DNase treated with the RNA-free DNase set (Qiagen)
193 according to manufacturer's instructions and quantified with the Nanodrop 1000
194 Spectrophotometer (Thermo Scientific). 1 μ g of each RNA was used for the cDNA
195 synthesis in a 20 μ L final volume reaction containing 200 U of SuperScript III Reverse
196 Transcriptase (Invitrogen) and 2.5 μ M oligo (dT) 20 primer (Invitrogen), following the
197 manufacturer's instructions.

198

199 2.4. Gene isolation

200 The *RiCRD1* gene sequence was previously identified by Tamayo *et al.* (2014) in
201 the *R. irregularis* genome available in the JGI website
202 (<https://genome.jgi.doe.gov/portal/>). The 5' and 3' ends were experimentally confirmed
203 by rapid amplification of cDNA ends (RACE) using the SMARTer® RACE 5'/3' kit
204 (Clontech) and the *RiCRD1*-specific primers listed in Table S2. Genomic clone and full-
205 length cDNA of *RiCRD1* were obtained by PCR amplification of *R. irregularis* genomic
206 DNA and cDNA, respectively, from ERM grown under control conditions in
207 monoxenic cultures, using a set of primers flanking the complete open reading frame
208 (Table S2). PCR products were cloned into pENTR/D-TOPO (Invitrogen) via TOPO
209 reaction. The full-length cDNA was then cloned into the yeast expression vector
210 pDRf1-GW (Addgene) by using the Gateway LR Clonase recombination system
211 (Invitrogen).

212 2.5. Sequence Analysis

213 Transmembrane domains of the protein were predicted using the TMHMM Server
214 v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). The E1-E2 ATPase, hydrolase and
215 heavy metal associated domains were identified via the Pfam Software v. 32.0
216 (<https://pfam.xfam.org/>). Additionally, CD-Search
217 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) was used to verify the presence
218 of the P-type ATPase Cu-like signature cd02094 (NCBI). These results were used to
219 generate a structural model of *RiCRD1* using MyDomains tool of Prosite
220 (<https://prosite.expasy.org/mydomains/>). Protein subcellular localization was predicted
221 by WoLF PSORT (<https://wolfsort.hgc.jp/>).

222 Additionally, *RiCRD1* full sequence was used as a query to identify orthologs
223 through Blastp searches in other Glomeromycotina species deposited on the JGI

224 (*Rhizophagus clarus* HR1, Kobayashi *et al.*, 2018; *Gigaspora rosea* v1.0, *Rhizophagus*
225 *cerebriforme* DAOM 227022 v1.0, *Rhizophagus diaphanus* v1.0, Morin *et al.*, 2019;
226 *Gigaspora margarita* BEG34, Venice *et al.*, 2020; *Geosiphon pyriformis*, Malar *et al.*,
227 2021) and NCBI websites. These sequences were aligned using Muscle v3.7 software
228 with the complete HMA family of *R. irregularis*, other HMA-like fungal proteins from
229 representatives of different taxonomic groups and the HMA proteins from the model
230 plants *Arabidopsis thaliana* and *Oryza sativa*. Alignments were imported to the IQ-
231 TREE software v1.6.12 (Nguyen *et al.*, 2015) with parameters -nt AUTO, -bb 1000 -m
232 TESTMERGE. The maximum likelihood tree was constructed following the model of
233 evolution LG+I+G4 (best-fit model according to ModelFinder; Kalyaanamoorthy *et al.*,
234 2017). Finally, the phylogenetic tree was plotted using the Interactive Tree of Life
235 (iTOL) suite software v4 (Letunic and Bork, 2016).

236 2.6. Functional complementation analyses in yeast

237 Metal hypersensitive yeast mutants *cup1Δ* and *yap1Δ* were transformed with the
238 resulting *RiCRD1* construct or with the corresponding empty vector (negative control)
239 using a lithium acetate-based method (Gietz and Schiestl, 2007). Transformants were
240 selected in SD medium by autotrophy to uracil. For drop tests, transformants were
241 grown to exponential phase in SD medium without uracil. Cells were harvested by
242 centrifugation, washed twice and adjusted to a final OD₆₀₀ of 1. Then, 5 μL of serial
243 1:10 dilutions were spotted on the corresponding selective medium. The transformed
244 *cup1Δ* cells were spotted onto SD medium without uracil supplemented or not with
245 75 μM CuSO₄ or with 100 μM CdSO₄ and *yap1Δ* cells onto SD medium without uracil
246 supplemented or not with 2 mM CuSO₄ or with 100 μM CdSO₄. Plates were incubated 5
247 days at 30 °C.

248

249 2.7. *Real-time quantitative RT-PCR*

250 Gene expression patterns were analyzed in an iQTM5 Multicolor Real-Time PCR
251 Detection System (Bio-Rad) using iQTM SYBR Green Supermix (Bio-Rad) and the
252 specific primers listed in Table S2. The program consisted in an initial incubation at
253 95°C for 3 min, followed by 38 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30
254 s, at the end of which the fluorescence signal was measured. The specificity of the PCR
255 amplification procedure was checked with a heat-dissociation protocol (from 58 to
256 95°C) after the final PCR cycle. Efficiency of the different primer sets was in the range
257 95-105 %. Since RNA extracted from mycorrhizal roots contains plant and fungal
258 RNAs, specificity of the primer pairs was also analyzed by PCR amplification of carrot
259 and chicory cDNA from non-mycorrhizal carrot and chicory roots. The relative
260 abundance of the transcripts was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen,
261 2001) and normalized with the *R. irregularis* elongation factor 1 α (*RiEF1 α* ; GenBank
262 Accession No. DQ282611; Benabdellah *et al.*, 2009). All determinations were
263 performed in at least three biological samples with the threshold cycle (Ct) determined
264 in duplicate in at least two independent PCRs.

265 2.8. *In situ hybridization of RiCRD1 transcripts in mycorrhizal roots*

266 200 bp sense and antisense probes of RiCRD1 and 18S RNA were generated by two
267 nested PCR reactions using gene-specific primers containing a 5' overhang to allow their
268 fusion to the T7 RNA polymerase promoter sequence (Table S2). The first PCR was
269 carried out on cDNA from ERM grown under control conditions in monoxenic cultures
270 with the primer pairs RiCRD1-T7-Pup and RiCRD1-Pdown or RiCRD1-Pup and
271 RiCRD1-T7-Pdown. The second PCR was performed using 1 μ L of a 1/100 dilution of
272 the amplicon and the primer pairs E-T7 and RiCRD1-Pdown or RiCRD1-Pup and E-T7.
273 Both amplifications were performed with GoTaq@G2 DNA polymerase (Promega) in a

274 final volume reaction of 25 μ L, using the protocol: 94°C for 3 min, followed by 40
275 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s. Amplification products were
276 purified by ethanol precipitation and used to obtain digoxigenin-UTP-labelled RNA
277 probes using the MAXIscript® T7 Transcription Kit following manufacturer's
278 instructions (Invitrogen). 18S sense and antisense ribosome probes were used as a
279 positive control (Garcia *et al.*, 2013).

280 Hybridization and detection of the probes were performed on 8 μ m-thick
281 sections of 8-week-old mycorrhizal tomato roots, as described in Jabnune *et al.* (2009).
282 Briefly, 3 mm root fragments were vacuum infiltrated in 4% (w/v) paraformaldehyde,
283 0.1% Triton X-100 in phosphate-buffered saline (10mM PBS), fixed overnight at 4°C
284 and embedded in paraffin (ParaplastPlus, Leica BioSystems). Longitudinal and cross-
285 sections of 8 μ m-thickness were obtained using a microtome Leica RM2255 and
286 mounted on silanized slides (Euromedex). Sections were deparaffinized with Safesolv
287 (Labonord), rehydrated and treated at 37°C for 40 min with proteinase K (0.1 U mL⁻¹).
288 To stop proteinase K activity, sections were washed twice for 5 min in an arrest buffer
289 (20 mM Tris-HCl pH 7.5, 2 mM CaCl₂, 50 mM MgCl₂), once for 2 min in PBS
290 containing 0.2% glycine and twice in PBS. Hybridizations were carried out in a humid
291 chamber at 45°C for 15 h on dehydrated sections using 600 ng of the corresponding
292 probe by slide, as described in Jabnune *et al.* (2009) including the stringency washes.
293 Non-linked probes were removed with 20 μ g mL⁻¹ RNase A for 30 min at 37°C.
294 Immunological detection of digoxigenin-labelled RNA hybrids was performed with
295 anti-digoxigenin antibodies conjugated with alkaline phosphatase enzyme (Roche),
296 following manufacturer's instructions. Finally, detection of hybridization signal was
297 performed using Vector Blue Alkaline Phosphatase Substrate kit (Vector Laboratories)
298 according manufacturer's instructions and images were taken on the Nikon Eclipse Ni-E

299 microscope (Nikon Corporation, Tokyo, Japan), objectives Plan APO 20x NA 0.75, 40x
300 NA 0.95 and 100x NA 1.45. An aliquot of the same root fragments was separated to
301 estimate mycorrhizal colonization.

302 2.9. Statistical Analyses

303 Statistical analyses were performed with IBM SPSS Statistic software v.25. Data
304 were subjected to the Student's t-test when two means were compared, or by one-way
305 ANOVA using post hoc comparison with Tukey's b-test to detect differences among
306 groups of means. Results were accepted as significant at $P < 0.05$. The data are
307 expressed as mean \pm standard error. All the analyses are based on at least 3 biological
308 replicates per each treatment ($n \geq 3$).

309 2.10. Gene Accession Numbers

310 GeneBank Accession numbers of the *R. irregularis* gene analyzed in this study:
311 *RiCRD1* (XM_025327727), *RiMT1*, formerly named *GintMT1* (XM_025308927),
312 *RiABC1*, formerly named *GintABC1* (GQ249346), *RiPCS* (XM_025316197); *RiMST2*
313 (HM143864).

314

315 3. Results

316 3.1. Sequence analyses of the Rhizophagus irregularis *RiCRD1* heavy metal ATPase

317 The full-length cDNA sequence of *RiCRD1* encodes a protein of 946 amino acids
318 (GenBank Accession No. XP_025169806). Comparison of the full-length cDNA with
319 the genomic sequence revealed the presence of two introns of 92 and 76 bp flanked by
320 the characteristic splicing sequences GT and AG at the 5' and 3' ends, respectively (Fig.
321 2). The *RiCRD1* protein contains all the characteristic features of P_{1B}-type (CP_X-type)
322 ATPases, including the conserved transmembrane cysteine-proline-cysteine motif

323 (CPC) that is essential for metal translocation. The protein contains eight
324 transmembrane helices with the CPCX₆P motif in the sixth transmembrane helix typical
325 of the P_{1B-1} subgroup of metal ATPases that transport Cu⁺ ions, two heavy metal
326 associated domains (PF00403) in the N-terminus, the E1-E2 ATPase domain
327 (PF00122), the hydrolase domain (PF00702) including the DKTGT phosphorylation
328 signature sequence, and the invariant histidine-proline HP dipeptide at 41 residues C-
329 terminal from the phosphorylation signature (Arguello, 2003; Arguello *et al.*, 2007;
330 Smith *et al.*, 2014; Solioz and Vulpe, 1996). The presence of the complete signature
331 (cd02094) characteristic of P-type ATPase Cu-like proteins was identified in the
332 RiCRD1 sequence, including the two cysteine residues CXC in the sixth transmembrane
333 helix; one tyrosine, one asparagine, and one proline YNX₄P residue in the seventh
334 transmembrane helix and one methionine followed by serine residues MXXSS in the
335 eighth transmembrane helix (Arguello, 2003) (Fig. 2). RiCRD1 was predicted to be
336 located at the plasma membrane, with the N- and C-termini facing the cytoplasmic side,
337 suggesting that RiCRD1 encodes a heavy metal ATPase that pumps excess Cu⁺ out of
338 the cytosol.

339 The phylogenetic analysis revealed that all fungal ATPases were clustered into two
340 different groups separated from those of plants: a CCC2-like group clustering orthologs
341 of the *S. cerevisiae* CCC2 Cu-ATPase (Yuan *et al.*, 1995) and a group of fungal
342 ATPases related to metal tolerance, which comprises two subgroups, the PCA1-like and
343 CRD1-like ATPases. The PCA1-like subgroup clusters orthologs of a Cd-efflux plasma
344 membrane ATPase of *S. cerevisiae* (Adle *et al.*, 2007) and the CRD1-like subgroup
345 includes orthologs of the *C. albicans* plasma membrane ATPase that exports excess of
346 Cu out of the cell (Yuan *et al.*, 1995). RiCRD1 is placed in the CRD1-like clade, which
347 suggests that it acts as a plasma membrane Cu efflux transporter. Blastp searches for

348 RiCRD1 homologues in the genomes of various Glomeromycotina species revealed that
349 the *R. irregularis* genome, as well as the genome of most Glomeromycotina species,
350 harbors one *CRD1*-like gene. However, two and three paralogues were identified in the
351 genomes of *Funneliformis caledonium* and *Claroideoglomus candidum*, respectively.
352 All Glomeromycotina CRD1 sequences were grouped together in the CRD1-like
353 subgroup (Fig. 3). Except for the two CRD1 sequences of *Dentiscutata erythropus*,
354 which have three heavy metal associated domains (PF00403), the Glomeromycotina
355 sequences have two (Table S3).

356 3.2. RiCRD1 encodes a functional protein involved in Cu tolerance

357 Due to the difficulty of gene manipulation in AM fungi, functionality of RiCRD1
358 was evaluated by a complementation assay in yeast. Since *S. cerevisiae* lacks CRD1
359 orthologs, functional analysis of RiCRD1 was carried out by testing the ability of the
360 full-length *RiCRD1* gene product to rescue metal sensitivity of the *cup1Δ* and *yap1Δ*
361 mutant strains of *S. cerevisiae*. CUP1 is a metallothionein that confers heavy metal
362 tolerance to yeast cells by sequestering metal ions in the cytosol via the thiol groups of
363 its cysteine residues (Ecker *et al.*, 1986; Hamer, 1986) and the transcription factor yap1
364 controls various genes involved in heavy metal and oxidative stress tolerance in yeast
365 (Kuge and Jones, 1994; Shine *et al.* 2015). Inactivation of yap-1 protein results in an
366 oxidative stress sensitive phenotype (Toone and Jones, 1999). The *cup1Δ* and *yap1Δ*
367 mutants are particularly sensitive to Cu and Cd and are, thus, suitable to highlight
368 tolerant phenotypes induced by exogenous cDNAs (Wu *et al.*, 1993). Copper
369 hypersensitivity of *cup1Δ* cells is due to their inability to sequester metal excess in the
370 cytosol, while Cu hypersensitivity of the *yap1Δ* mutant results from the oxidative stress
371 caused by the accumulation of free Cu in the cytosol. As shown in Fig. 4, *RiCRD1*-
372 expressing cells enhanced Cu tolerance of *cup1Δ* and *yap1Δ* strains when grown in

373 media containing 75 μM and 2 mM of CuSO_4 , respectively. These data indicate that
374 *RiCRD1* encodes a functional protein that confers Cu tolerance to yeast cells.

375 Since the CaCRD1 ortholog of *C. albicans* has also been shown to be involved in
376 resistance to Cd ion toxicity (Riggle and Kumamoto, 2000), we also tested whether
377 RiCRD1 could additionally confer some kind of Cd protection to these mutant strains.
378 However, either empty vector-transformed cells or those expressing *RiCRD1* were
379 unable to grow in SD medium supplemented with a gradient of CdSO_4 concentrations
380 up to 100 μM (data not shown).

381 3.3. *RiCRD1* expression is up-regulated in response to high concentration of Cu and
382 Cd in the medium

383 To investigate whether RiCRD1 could play a role in metal tolerance by detoxifying
384 Cu excess out of the fungus, *RiCRD1* gene expression was assessed by real time
385 quantitative RT-PCR (RT-qPCR) in ERM grown in monoxenic cultures under different
386 Cu (250 and 500 μM) levels. As previously observed by Cornejo *et al.* (2013), some
387 blue spores indicative of Cu compartmentalization were observed in ERM 2 days after
388 Cu addition to the medium (Fig. 5A). Exposure of the mycelia to high Cu levels
389 increased transcription of *RiCRD1* at all the time points analyzed (Fig. 5A). This
390 increase in transcript accumulation reaches 25-30 times the control level in response to
391 increasing Cu concentration in the medium and time. These results are consistent with a
392 role of *RiCRD1* in Cu detoxification.

393 *RiCRD1* transcript levels were also determined in monoxenically grown ERM
394 exposed to 45 μM CdSO_4 for different time periods. Interestingly, in contrast to elevated
395 Cu levels, *RiCRD1* expression was only transiently induced by Cd. A 3-fold induction
396 was observed 3 and 6 h after Cd addition, followed by a significantly decrease in gene
397 expression (Fig. 5B).

398 3.4. *RiCRD1* is a major player in *R. irregularis* Cu tolerance

399 To get some clues about the significance of *RiCRD1* on metal tolerance in *R.*
400 *irregularis*, the *RiCRD1* transcript accumulations in Cu- and Cd- treated ERM were
401 compared with the transcript accumulations of other Cu- and Cd-responsive genes
402 previously identified in the *R. irregularis* genome: the metallothionein *RiMT1*,
403 (González-Guerrero *et al.*, 2007), the ABC-transporter *RiABC1* (González-Guerrero *et*
404 *al.*, 2010), and the phytochelatin synthase *RiPCS* (Shine *et al.*, 2015). Transcript levels
405 of *RiMT1* were not significantly affected by Cu, except in ERM exposed for 2 d to 500
406 μM CuSO_4 (Fig. 6A). In contrast, ERM exposure to 45 μM Cd resulted in a stable 2- to
407 5-fold down-regulation of *RiMT1* 12 to 48 h after the application of the treatment (Fig.
408 6B). *RiABC1* transcript levels were only significantly changed 2 and 7 days after ERM
409 exposure to 500 μM CuSO_4 (2-fold increase) and 6 h after ERM exposure to 45 μM Cd
410 (transient 5-fold increase) (Figs. 6C, D). Interestingly, *RiPCS* transcript accumulation
411 was 2-fold reduced in response to Cu exposure but unchanged in response to Cd
412 exposure (Figs. 6E, F). Therefore, the expression of *RiCRD1* was much more impacted
413 in response to Cu than other metal regulators of the intracellular metal levels.

414 3.5. *RiCRD1* is more highly expressed in the intraradical mycelium

415 To further understand the role that *RiCRD1* could play in *R. irregularis* and in the
416 symbiosis, we assessed its expression level in the ERM and IRM grown under optimal
417 conditions in two experimental systems: monoxenic cultures and the *in vivo* whole plant
418 bidimensional experimental system (sandwich system). Transcript levels of the *R.*
419 *irregularis* high-affinity monosaccharide transporter *RiMST2*, which is strongly up-
420 regulated in the IRM during AM symbiosis (Helber *et al.*, 2011), was also determined
421 as a marker of fungal activity. Carrot roots collected from the monoxenic cultures
422 presented 10% of mycorrhizal colonization while the percentage of mycorrhizal

423 colonization of the chicory roots used to grow the fungus in the sandwich system was
424 78%. In both experimental systems, *RiMST2* and *RiCRD1* were more highly expressed
425 in the IRM than in the ERM. *RiCRD1* transcript levels were 18-fold higher in carrot
426 mycorrhizal roots than in ERM collected from monoxenic cultures and 25-fold higher in
427 mycorrhizal chicory roots than in ERM collected from the *in vivo* sandwich system
428 (Fig. 7). This expression pattern hints at the importance of *RiCRD1* in the intraradical
429 phase of the fungus, where it might mediate the efflux of Cu from the fungus to the
430 apoplast of the symbiotic interface.

431 3.6. *RiCRD1* is expressed in the arbuscules

432 Given that arbuscules developed in plant cortical cells are the main structures where
433 nutrient exchanges between symbionts take place, Cu transfer from the fungus to the
434 plant should occur in the arbuscule-colonized cortical cells (Luginbuehl and Oldroyd,
435 2017; MacLean *et al.*, 2017). However, since the fungus develops other intraradical
436 structures, we decided to determine the specific fungal structure where *RiCRD1* is
437 expressed by performing an *in situ* hybridization assay in tomato roots presenting a 40%
438 of mycorrhizal colonization (Figs. 8A-B). As a positive control of hybridization and
439 RNA quality, expression of the 18S ribosomal gene was monitored (Fig. S1). *RiCRD1*
440 transcripts were clearly detected with the antisense probe in the arbuscules developed in
441 the inner cortical cells while no signal was detected in any other fungal structure. This
442 expression pattern indicates that arbuscules are likely the sites of Cu efflux (Figs. 8C-F).

443 3.7. Expression of *RiCRD1* decreases in conditions of Cu limitation mycorrhizae 444 generated in Cu-deprived media

445 To test whether *RiCRD1* expression is affected by Cu availability, we assessed
446 the influence of growing the roots under Cu-limiting conditions on the transcription of

447 *RiCRD1*. For this purpose, transcript accumulation of *RiCRD1* was determined by RT-
448 qPCR in *R. irregularis* colonized roots grown in monoxenic cultures and in the *in vivo*
449 sandwich system in the presence (control) and absence of Cu (Cu deficiency). Cu
450 deficiency decreased mycorrhizal colonization of the carrot and chicory roots
451 developed in the monoxenic and *in vivo* cultures, respectively, in comparison to control
452 conditions, which was confirmed molecularly by the quantification of the amount of the
453 fungus within the root (Table S4). Accumulation of *RiCRD1* transcripts was lower in
454 mycorrhizal roots grown in conditions of Cu limitation than in control conditions (0.5
455 μM Cu in monoxenic cultures and 0.16 μM in the *in vivo* sandwich system) (Fig. 9).
456 These data suggest that Cu efflux from the fungus is reduced under Cu-limiting
457 conditions.

458

459 4. Discussion

460 AM fungi play an important role in modulating plant Cu acquisition in a wide range
461 of Cu concentrations. The potential of AM fungi to either increase plant Cu uptake in
462 poor Cu soils or alleviate Cu toxicity has led to the hypothesis that AM function as a
463 “buffer” to protect the plant against damage produced by lack or excess of Cu in the soil
464 (Ferrol *et al.*, 2016; Gómez-Gallego *et al.*, 2019). Here, we report characterization of
465 *RiCRD1*, a *R. irregularis* gene encoding a protein with a role in Cu tolerance, which,
466 according to its sequence features, is most likely a plasma membrane Cu-ATPase.

467 4.1. Identification of *RiCRD1* as a putative Cu-ATPase with a role in Cu tolerance

468 *In silico* analysis of the *RiCRD1* protein and expression patterns of *RiCRD1*
469 when the ERM was exposed to high Cu levels strongly suggest that *RiCRD1* is the
470 ortholog of the plasma membrane Cu efflux P_{1B1}-type ATPase CaCRD1 of *C. albicans*

471 (Riggle and Kumamoto, 2000; Weissman *et al.*, 2000). RiCRD1 has all the
472 characteristic features of P_{1B}-type ATPases, and more specifically of those belonging to
473 the P_{1B-1} subgroup, including the complete cd2094 signature of Cu-like proteins that
474 transport Cu⁺ ions and the invariant CPCX₆P motif typical of the P_{1B-1} subgroup
475 (Arguello, 2003). It contains eight transmembrane domains, with the CPC motif that is
476 needed for metal translocation in the sixth transmembrane helix (Arguello *et al.*, 2007).
477 Additionally, the invariant HP dipeptide was found 40 residues downstream to the
478 phosphorylation site. Although the function of this motif is still unknown, it seems to
479 have some relevance since replacement of the histidine by a glutamic acid induces
480 abnormalities of copper metabolism in the Wilson's disease (Bissig *et al.*, 2001; Solioz
481 and Vulpe, 1996; Tanzi *et al.*, 1993). Interestingly, RiCRD1 has two heavy metal
482 associated domains in the N-terminus, although only one strictly has the classical
483 GMXCXXC motif. The first domain, GLTCASC, has the CXXC motif characteristic of
484 proteins that bind copper (Camakaris *et al.*, 1999; Migocka, 2015; Smith *et al.*, 2014;
485 Strausak *et al.*, 1999), but the second methionine is changed by a leucine. The N-
486 terminus of RiCRD1, as well as most of the Glomeromycotina CRD1-like sequences,
487 presents a reduced number of metal binding domains in comparison with other
488 eukaryote Cu-ATPases, which usually have multiple repeats of this domain (Arguello *et*
489 *al.*, 2007; Rensing *et al.*, 1999). For instance, CaCRD1 has five metal binding domains,
490 including two CXXC and three GMXCXXC motifs (Riggle and Kumamoto, 2000;
491 Weissman *et al.*, 2000). However, numerous prokaryotic Cu-transporting ATPases have
492 a single N-terminal metal binding domain (Rensing *et al.*, 2000). A reduced number of
493 CXXC N-terminal repeats seems to be a characteristic feature of Glomeromycotina
494 CRD1 proteins. These N-terminal metal binding domains of P_{1B-1} subgroup are
495 homologous to a number of metal chaperone proteins, can bind Cu⁺, Cu²⁺, Zn²⁺, Cd²⁺

496 and exchange metals with the related chaperons. A regulatory role rather than an
497 essential catalytic role has been proposed for these N-terminus metal binding domains
498 (Arguello, 2003).

499 Our yeast heterologous complementation assays show that *RiCRD1* encodes a
500 protein with a role in Cu tolerance, as it was able to protect the metal hypersensitive
501 yeast *cup1Δ* and *yap1Δ* mutants against Cu toxicity. Since Cu hypersensitivity of both
502 yeast strains results from Cu overaccumulation in the cytosol, our complementation
503 assays indicate that, at least in the heterologous system, RiCRD1 decreases Cu levels in
504 the cytosol. Previously characterized P_{1B}-type ATPases from fungi and prokaryotes
505 involved in Cu homeostasis exhibit tightly controlled transcriptional regulation
506 consistent with their physiological roles (Arguello, 2003; Antsoategi-Uskola *et al.*, 2017;
507 Antsoategi-Uskola *et al.*, 2020; Benes *et al.*, 2018; Wiemann *et al.* 2017). The strong up-
508 regulation of *RiCRD1* in the ERM in response to high Cu concentration in the medium
509 supports the notion that RiCRD1 can play a role in Cu detoxification in *R. irregularis*
510 ERM. These observations, together with the structural features and predicted plasma
511 membrane location of RiCRD1, strongly suggest a role for RiCRD1 in Cu efflux from
512 the cytosol.

513 4.2. *RiCRD1* and Cu tolerance in *R. irregularis*

514 RiCRD1 would enable the fungus to avoid the accumulation of intracellular toxic
515 levels of Cu by facilitating Cu efflux through the plasma membrane. This hypothesis is
516 supported by the low cytoplasmic concentrations of Cu detected in the *R. irregularis*
517 ERM when exposed to high Cu levels (González-Guerrero *et al.*, 2008). Our data
518 showing that in the ERM subjected to the highest Cu concentrations *RiMT1* expression
519 was just slightly and transiently induced, while *RiCRD1* was highly up-regulated
520 suggests that *R. irregularis* uses the Cu efflux RiCRD1 pump as primary mechanism to

521 overcome Cu toxicity. Actually, the role of the *R. irregularis* metallothionein RiMT1 in
522 Cu tolerance was attributed to its antioxidant activity against the metal-induced
523 oxidative stress rather than on its metal chelation activity (González-Guerrero *et al.*,
524 2007). These results are in agreement with those described in *C. albicans* and some
525 filamentous fungi, such as *Aspergillus nidulans* (Antsoetegi-Uskola *et al.*, 2020; Riggle
526 and Kumamoto, 2000; Weissman *et al.*, 2000), but in contrast with what happens in *S.*
527 *cerevisiae*, in which Cu resistance mainly relies on Cu chelation by the CUP1
528 metallothionein (Ecker *et al.*, 1986; Thiele, 1988). Here, we propose for the first time
529 that AM fungi use a Cu efflux strategy to cope with Cu toxicity. In addition to this Cu
530 efflux strategy, as previously reported by Cornejo *et al.* (2013), *R. irregularis*
531 compartmentalizes part of the excess Cu in some spores of the fungal colony, as some
532 blue spores indicative of Cu accumulation were observed in some of the Cu-exposed
533 ERM.

534 4.3. Cd tolerance in *R. irregularis*

535 In *C. albicans*, *CRD1* null mutants presented increased sensitivity not only to Cu but
536 also to Cd ions (Riggle and Kumamoto, 2000). This raised the question of whether
537 RiCRD1 could also have a secondary role in Cd resistance in *R. irregularis*. *RiCRD1*
538 expression was up-regulated in the ERM exposed to Cd, although this induction was
539 faster, transient and less intense than with Cu. However, failure of RiCRD1 to recover
540 the phenotype of the yeast metal hypersensitive mutants *cup1Δ* and *yap1Δ* in media
541 supplemented with Cd rules out a function for RiCRD1 in protection against Cd
542 toxicity. These data suggest that RiCRD1 cannot transport Cd²⁺ ions, which is
543 consistent with the fact that the P_{1B-1} subgroup of P_{1B}-ATPases are highly specific for
544 the transport of monovalent Cu ions, the dominant intracellular species in eukaryotes
545 (Nevitt *et al.*, 2012). Transient accumulation of *RiCRD1* transcripts during the early

546 stages of Cd exposure could be caused by disturbed metal homeostasis with transient
547 increase in cytosolic Cu. Alternatively, as previously stated by Antsoategi-Ukola *et al.*
548 (2017), some kind of interaction of CRD1-like proteins with Cd stress might take place
549 transiently when other more specific pathways for Cd detoxification are saturated.

550 Down-regulation of *RiMT1* expression by Cd indicates that Cd detoxification should
551 rely on other specific players and agrees with previous hypothesis that metallothioneins
552 do not constitute the primary control point for metal detoxification in *R. irregularis*
553 (González-Guerrero *et al.*, 2007). Other candidate players of metal detoxification in *R.*
554 *irregularis* could be phytochelatins, small peptides synthesized enzymatically from
555 glutathione by phytochelatin synthase that form complexes with metals in the
556 cytoplasm, which are then transported into the vacuoles (Cobbett, 2000a; Heiss *et al.*,
557 2003; Mendoza-Cozatl *et al.*, 2010). The *R. irregularis* genome encodes a phytochelatin
558 synthase (*RiPCS*) (Shine *et al.*, 2015) that is not transcriptionally regulated by Cd.
559 Although PCSs were considered to be sparsely distributed in the fungal kingdom, a
560 recent analysis of the distribution of candidate PCS in fungal genomes reveals their
561 presence in many lineages (Shine *et al.*, 2015). However, PCS are usually expressed
562 constitutively and activated post-translationally by various essential and non-essential
563 metals, being Cd the most effective (Bolchi *et al.*, 2011; Pal and Rai, 2010). Despite the
564 regulatory mechanisms of PCS function remain elusive, it has been proposed that either
565 the metal alone or the GSH-metal complexes formed in the cytosol can interact with the
566 PCS cysteine residues (Cobbett, 2000b). Up-regulation by Cu and Cd of *RiABC1*, a gene
567 putatively encoding an ABC transporter that could be involved in metal transport into
568 the vacuoles (González-Guerrero *et al.*, 2010; Rekha *et al.*, 2021) suggests that long-
569 term acclimation to high levels of Cd would be achieved through metal accumulation
570 into the fungal vacuoles (González-Guerrero *et al.*, 2008; Park *et al.* 2012; Rekha *et al.*,

571 2021; Song *et al.*, 2014). However, further analyses are required to elucidate the role of
572 RiCRD1 in the early response to Cd toxicity and to decipher the mechanisms of Cd
573 tolerance in *R. irregularis*.

574 4.4. Nutritional and ecological relevance of RiCRD1

575 The finding that *RiCRD1* was strongly expressed in the intraradical fungal structures
576 and more specifically in the arbuscules hints at the importance of this protein for the
577 symbiosis. We propose that the putative Cu efflux pump RiCRD1 could be involved in
578 Cu release from the arbuscules to the apoplast of the symbiotic interface. However,
579 silencing of this gene by either host-induced gene silencing (HIGS) or virus-induced
580 gene silencing (VIGS) is needed to confirm this hypothesis. Down-regulation of
581 *RiCRD1* in the IRM by Cu deficiency suggests that under these conditions the fungus
582 reduces Cu efflux out of the cytosol. In fact, the decrease in *RiCRD1* transcript
583 accumulation in the IRM under Cu-limiting conditions could mean that the fungus
584 restricts the transfer of Cu to the plant in order to satisfy its own demand. This
585 hypothesis is supported by our previous observations that transcript levels of the plasma
586 membrane Cu uptake transporter *RiCTR1* increase under Cu deficient conditions and
587 that under these conditions the number of arbuscules is reduced (Gómez-Gallego *et al.*,
588 2019). Further physiological studies using radioactively labeled Cu and compartmented
589 pot systems with separate soil zones for hyphal growth combined with molecular
590 studies are required to understand the contribution and regulation of the mycorrhizal Cu
591 uptake pathway under different Cu supplies. Blastp searches in Glomeromycotina
592 species revealed at least one *CRD1*-like gene in all the examined species, suggesting
593 that this Cu efflux mechanism must not be unique to *R. irregularis*, and it is probably
594 shared by other AM fungi. Interestingly, *Funneliformis caledonium* displays two
595 paralogs and *Claroideoglossum candidum* three. More than one *CRD1*-like gene copy

596 has been described in other fungi such as in *Aspergillus* spp. which, if functional, might
597 provide some sort of adaptive advantage to their respective ecological niches as a result
598 of increased Cu export efficiency (Yang *et al.*, 2018).

599 On the other hand, we have recently shown that AM increases expression of HMA
600 genes putatively encoding proteins involved in Cu detoxification and balances mineral
601 nutrient uptake improving nutritional status of maize plants grown in Cu contaminated
602 soils (Gómez-Gallego *et al.*, 2022). Therefore, all these results together indicate that
603 AM fungi are able not only to up-regulate their own intrinsic Cu detoxification
604 mechanisms but also those of their host plants and highlight the importance of the HMA
605 genes to achieve balanced Cu levels. A better understanding of Cu transport
606 mechanisms by both partners could help to fine-tune their management in agricultural
607 fields to achieve more sustainable systems including the development of metal
608 alleviation strategies in metal contaminated soils.

609

610 5. Conclusions

611 In conclusion, data presented in this work show that the *R. irregularis* gene *RiCRDI*
612 encodes a protein with a role in Cu tolerance, which most likely is a plasma membrane
613 Cu-ATPase. This Cu⁺ exporting P-type ATPase could have a major impact not only on
614 metal detoxification but also on Cu transport through the mycorrhizal pathway by
615 releasing Cu into the apoplast of the symbiotic interface. Although this study represents
616 a breakthrough in the understanding of Cu homeostasis in AM fungi, further studies are
617 necessary to fully understand this complex Cu homeostatic network, which allows AM
618 fungi to maintain Cu intracellular levels balanced in a wide range of environments.

619

620 Author contributions

621 Tamara Gómez-Gallego: Conceptualization, Methodology, Formal analysis,
622 Investigation, Writing – original draft & editing. M^a Jesús Molina-Luzón: Methodology
623 & Investigation. Genevieve Conéjéro: Methodology, Supervision, Writing – review &
624 editing. Pierre Berthomieu: Methodology, Supervision, Writing – review & editing.
625 Nuria Ferrol: Conceptualization, Methodology, Supervision, Funding acquisition,
626 Project administration, Writing – original draft & editing.

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631 Data statement

632 All gene sequences used in this study are available in GenBank or JGI databases as
633 detailed, any further information can be provided by the corresponding author upon
634 reasonable request.

635 Declaration of competing interest

636 The authors declare no conflict of interest.

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641

642 **Appendix A. Supplementary data**

643 Supplementary data to this article can be found online.

644 Table S1: *Saccharomyces cerevisiae* strains used in this work, Table S2: Primers used
645 in this study, Table S3: CRD1-like sequences identified in Glomeromycotina species,
646 Table S4: Effect of Cu limitation on mycorrhizal colonization, Fig. S1: Controls used in
647 the *in situ* hybridization experiment.

648

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1012 **Figure captions**

1013 **Fig. 1.** Scheme of the two experimental systems used to grow *Rhizophagus irregularis*.

1014 (A) Monoxenic cultures established with transformed carrot roots (Ri T-DNA) in two-
1015 compartment Petri dishes containing M medium (St-Arnaud *et al.*, 1996) (*in vitro*
1016 culture system). (B) In vivo whole plant bidimensional experimental system established
1017 with chicory seedlings according to Pepe *et al.* (2017) with some modifications as
1018 detailed in Materials and Methods (sandwich system). CH: hyphal compartment; RC:
1019 root compartment; A: arbuscule; BAS: Branched Absorbing Structures; S: spores;
1020 ERM: extraradical mycelium; IRM: intraradical mycelium.

1021 **Fig. 2.** Schematic representation of the structure of *R. irregularis* RiCRD1 depicting the
1022 position of characteristic features of P_{1B}-type ATPases. This model was generated with
1023 the MyDomains tool of Prosite (<https://prosite.expasy.org/mydomains/>) based on the
1024 results of the TMHMM Server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) and
1025 the Pfam Software v. 32.0 (<https://pfam.xfam.org/>). Exon/intron organization of the
1026 *RiCRD1* genomic sequence, introns were illustrated with striped boxes and flanked by
1027 the canonical splicing sequences GT and AG at 5' and 3' ends, respectively.

1028 **Fig. 3.** Phylogenetic relationships of HMA proteins. *R. irregularis* HMA proteins are in
1029 bold and GenBank accession numbers of all the protein sequences used for the analyses
1030 are provided. The maximum likelihood tree was constructed following the model of
1031 evolution LG+I+G4 for amino acid sequences in IQ-TREE software. Colors of the
1032 branches represent levels of significance obtained in the bootstrapping analyses to
1033 define each cluster, as indicated in the figure legend (1000 bootstrap replicates).
1034 Organisms: Ac, *Acaulospora colombiana*; Af, *Aspergillus fumigatus*; Afl, *Aspergillus*
1035 *flavus*; And, *Aspergillus nidulans*; An, *Aspergillus niger*; As, *Amanita strobiliformis*;
1036 At, *Arabidopsis thaliana*; Bc, *Botrytis cinerea*; Cc, *Coprinopsis cinerea*; Cp,

1037 *Cetraspora pellucida*; Cg, *Colletotrichum gloeosporioides*; Clc, *Claroideoglo-*
 1038 *candidum*; Cn, *Cryptococcus neoformans*; De, *Dentiscutata erythropus*; Dh,
 1039 *Dentiscutata heterogama*; Deb, *Diversispora eburnea*; Dep, *Diversispora epigaea* Fc,
 1040 *Funneliformis caledonium*; Gp, *Geosiphon pyriformis*; Gr, *Gigaspora rosea*; Gm,
 1041 *Gigaspora margarita* Lb, *Laccaria bicolor*; Nc, *Neurospora crassa*; Pi, *Piriformospora*
 1042 *indica*; Pg, *Puccinia graminis*; Os, *Oryza sativa*; Rp, *Racocetra persica*; Rc,
 1043 *Rhizophagus cerebriforme*; Rcl, *Rhizophagus clarus*; Rd, *Rhizophagus diaphanous*; Ri,
 1044 *Rhizophagus irregularis*; Ro, *Rhizopus oryzae*; Sc, *Saccharomyces cerevisiae*; Sl,
 1045 *Suillus luteus*; Tm, *Tuber melanosporum*; Um, *Ustilago maydis*; Zm: *Zea mays*.

1046 **Fig. 4.** Functional analysis of RiCRD1 in metal hypersensitive yeast mutants. The
 1047 *Saccharomyces cerevisiae cup1Δ* and *yap1Δ* mutants were transformed with the empty
 1048 vector or expressing *RiCRD1* and plated on SD media supplemented or not with 75μM
 1049 and 2 mM CuSO₄, respectively. Plates were incubated 5 days at 30 °C.

1050 **Fig. 5.** Effect of high concentrations of Cu and Cd on *RiCRD1* transcript level in ERM.
 1051 *R. irregularis* ERM was grown in monoxenic cultures in M-C medium (control) or in
 1052 M-C medium supplemented with 250 μM CuSO₄, 500 μM CuSO₄ (A) or with 45 μM
 1053 CdSO₄ (B) and incubated at 24°C. The time of Cu or Cd addition was referred as time 0.
 1054 Mycelia were collected 1, 2 and 7 days after Cu addition and 1, 3, 6, 12, 24 and 48
 1055 hours after Cd supplementation. Some blue spores indicative of Cu
 1056 compartmentalization (pointed with blue arrows) were observed 2 days after Cu
 1057 addition to the ERM; images were captured under a binocular microscope just before
 1058 the collect of the ERM subjected to the different Cu treatments (Scale bar: 500 μm).
 1059 *RiCRD1* transcript levels were calculated by the 2^{-ΔΔCT} method using *RiEF1α* as a
 1060 normalizer. Bars represent standard error; different letters indicate statistically

1061 significant differences between treatments at the level of 0.05 according to the Tukey's
1062 b-test.

1063 **Fig. 6.** Effect of high concentrations of Cu and Cd on the transcript levels of metal
1064 tolerance related genes of *R. irregularis*. ERM was grown in monoxenic cultures in M-
1065 C medium (control) or in M-C medium supplemented with 250 μM CuSO_4 , 500 μM
1066 CuSO_4 or 45 μM CdSO_4 and incubated at 24°C. The time of Cu or Cd addition was
1067 referred as time 0. Mycelia were collected 1, 2 and 7 days after Cu addition and 1, 3, 6,
1068 12, 24 and 48 hours after Cd supplementation. Transcripts levels of (A-B) *RiMTI*, (C-
1069 D) *RiABC1*, and (E-F) *RiPCS* were calculated by the $2^{-\Delta\Delta\text{CT}}$ method using *RiEF1 α* as a
1070 normalizer. Bars represent standard error; different letters indicate statistically
1071 significant differences between treatments at the level of 0.05 according to the Tukey's
1072 b-test.

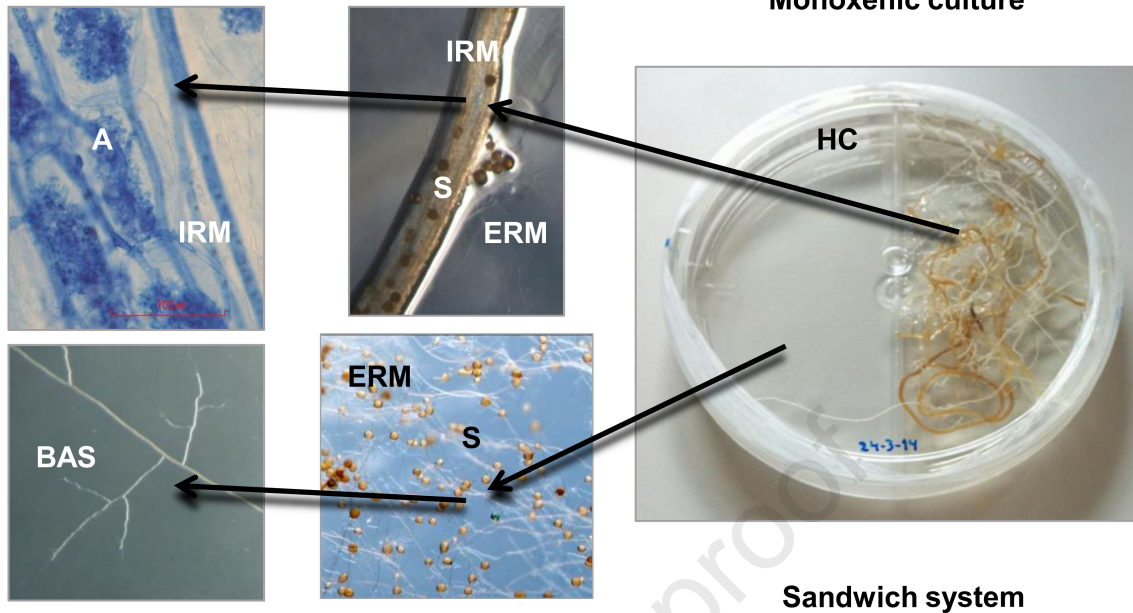
1073 **Fig. 7.** *RiCRD1* transcript levels in the *R. irregularis* ERM and IRM. Transcript levels
1074 of *RiCRD1* (A) and *RiMST2* (B) were measured in the extraradical mycelia (ERM) and
1075 the intraradical mycelia (IRM) of *R. irregularis* grown under control conditions in
1076 monoxenic cultures (i) in the presence of T-DNA transformed carrot roots (*in vitro*
1077 system) or (ii) in the whole plant bidimensional experimental system with chicory
1078 plants (*in vivo* system). Relative transcript levels were calculated by the $2^{-\Delta\Delta\text{CT}}$ method
1079 using *RiEF1 α* as a normalizer. The transcript level measured in the ERM was
1080 designated as 1. Bars represent standard error; * statistically significant differences at
1081 the level of 0.05 according to the Student's t-test.

1082 **Fig. 8.** Localization of *RiCRD1* transcripts by *in situ* hybridization in tomato roots (*L.*
1083 *esculentum* cv. Moneymaker) 8 weeks after mycorrhization with *R. irregularis*. (A-B)
1084 Trypan blue staining of roots showing root anatomy and arbuscules at two
1085 magnifications (C-F) Four repeats of the hybridization with the *RiCRD1* antisense probe

1086 showing a specific blue staining in arbuscules. (G-H) Two repeats of the hybridization
1087 with the *RiCRDI* sense probe, in which only a weak background signal was detected. a:
1088 arbuscules (see red arrows), c: cortical cells, v: vascular tissues. Scale bars represent 20
1089 μm .

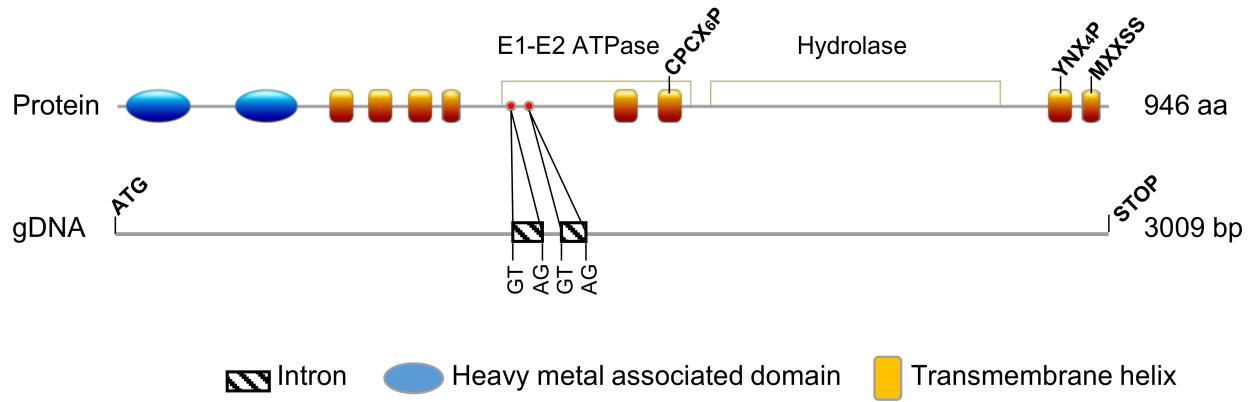
1090 **Fig. 9.** Effect of Cu deficiency on *RiCRDI* transcript levels in the IRM. *R. irregularis*
1091 colonized roots were grown in presence and in the absence of Cu in two experimental
1092 systems. Mycorrhizal carrot roots were grown in monoxenic cultures in M media
1093 (control, 0.5 μM Cu) or in M media lacking Cu (*in vitro* system) and mycorrhizal
1094 chicory roots were grown in the whole plant bidimensional experimental system (*in vivo*
1095 sandwich system) fertilized with half-strength Hoagland solution (control, 0.16 μM Cu)
1096 or with a modified nutrient solution without Cu. *RiCRDI* transcript levels were
1097 calculated by the $2^{-\Delta\Delta\text{CT}}$ method using *RiEF1 α* as a normalizer. Bars represent standard
1098 error; * statistically significant differences in comparison to the control value at the
1099 level of 0.05 according to the Student's t-test.

A

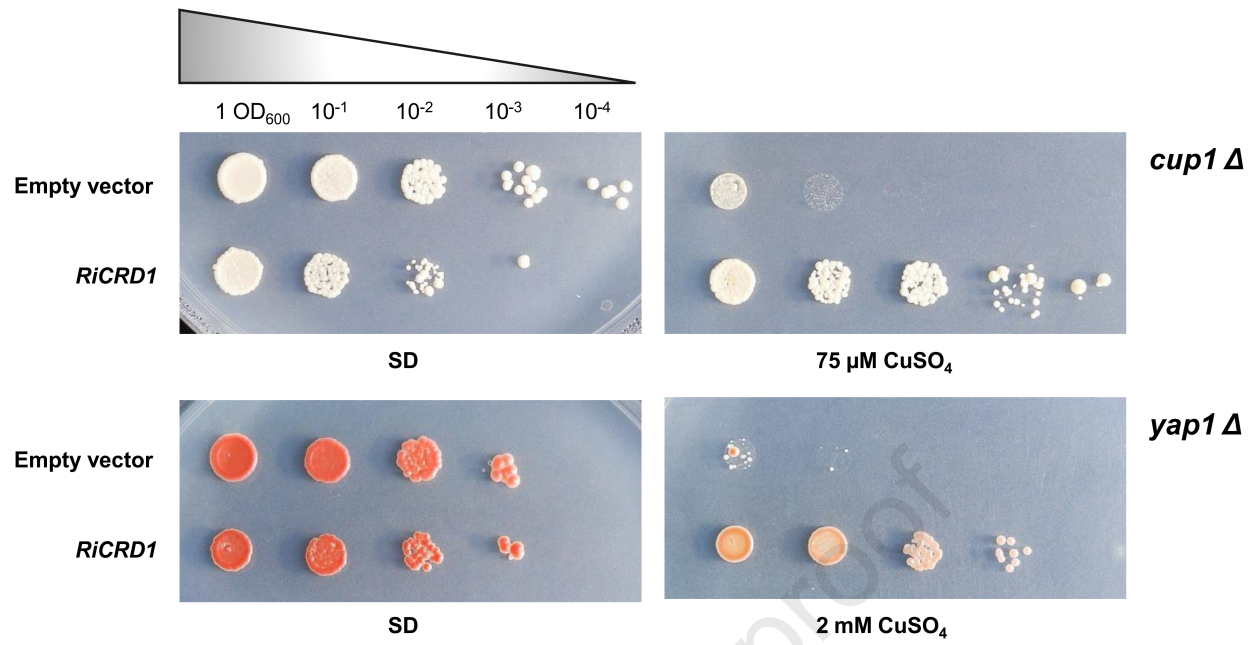


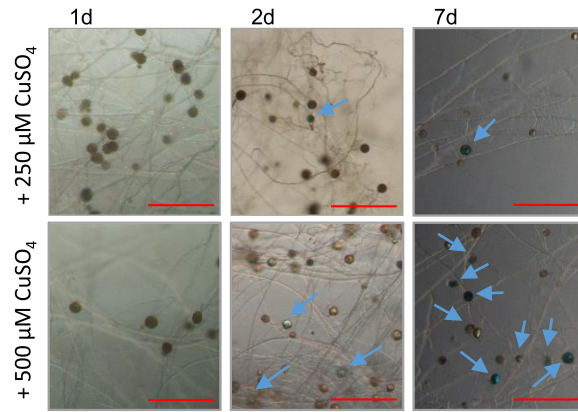
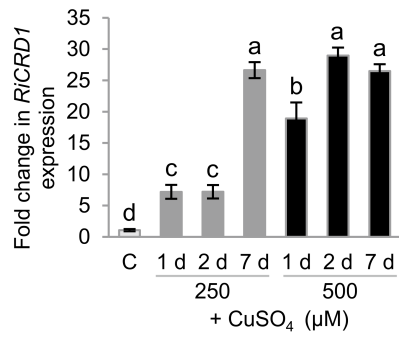
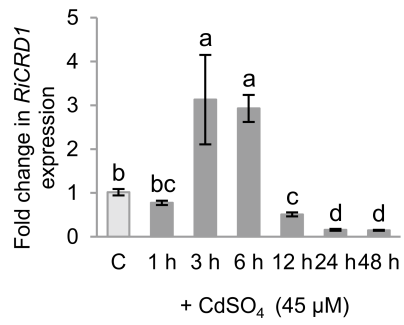
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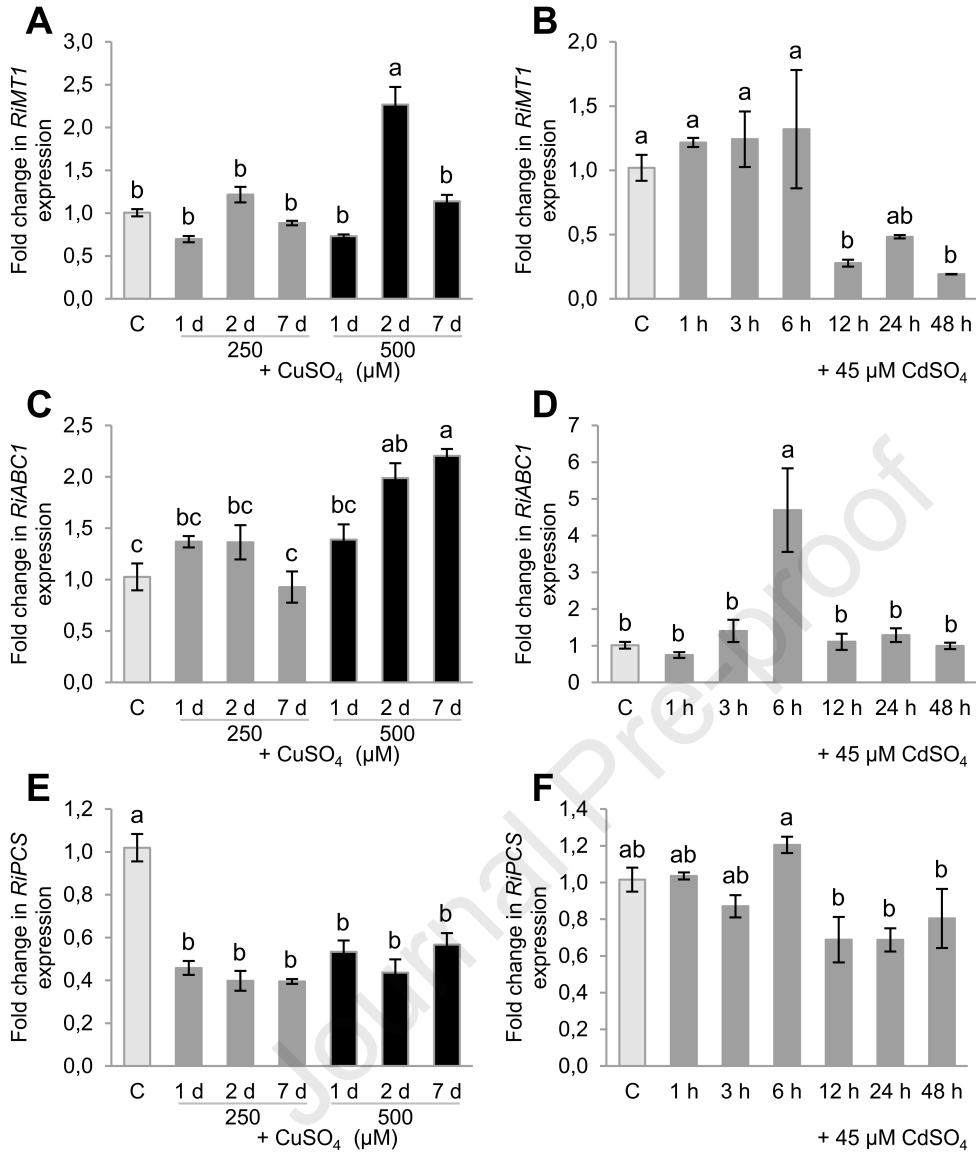


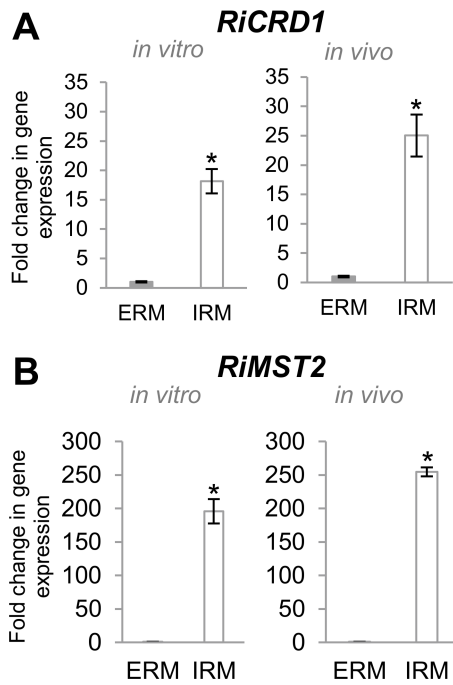
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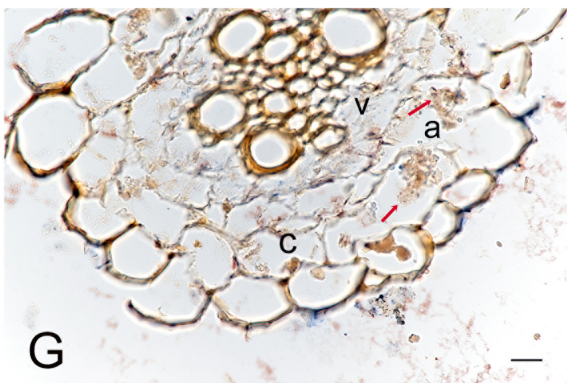
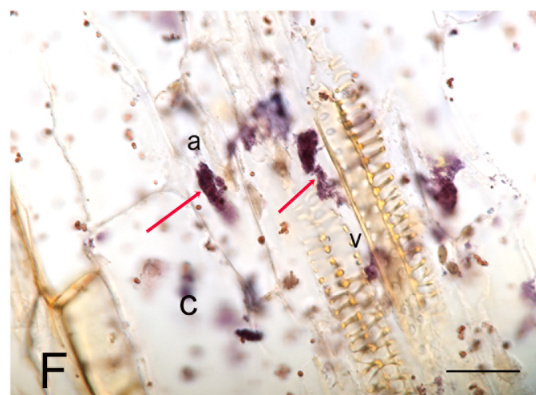
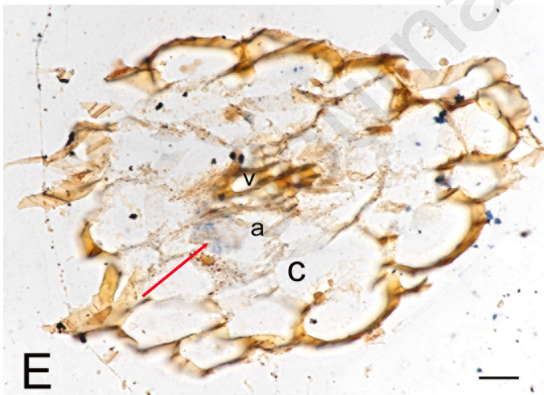
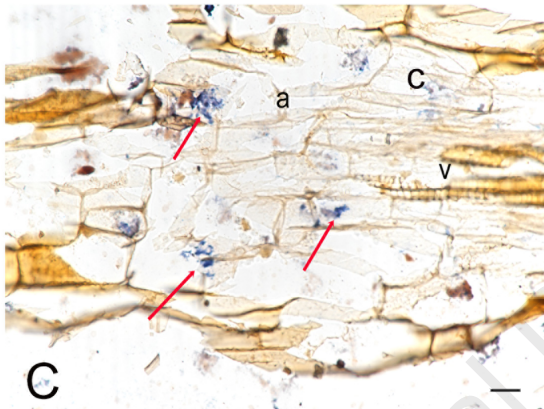
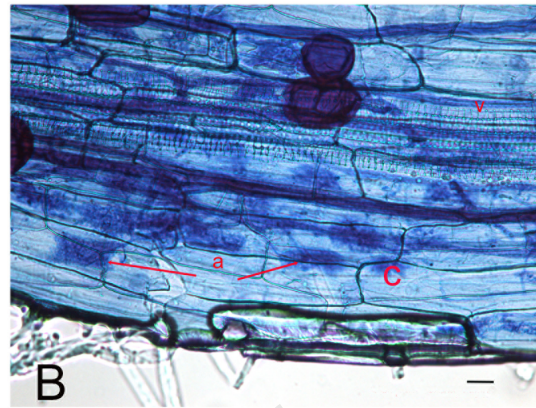
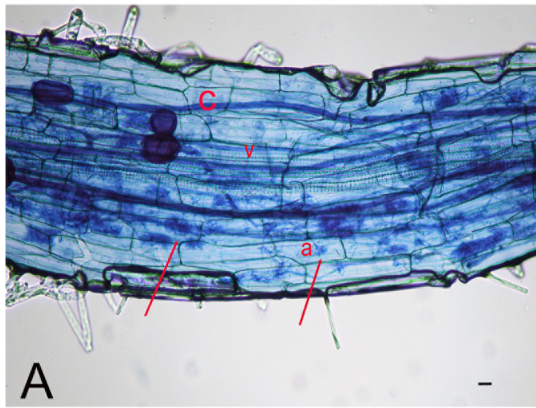
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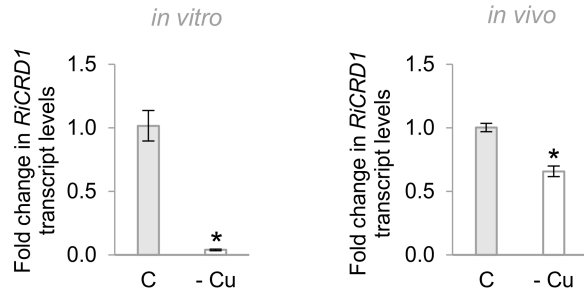




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- *RiCRD1* encodes a Cu exporting ATPase in *Rhizophagus irregularis*
- RiCRD1 could play a dual role in Cu detoxification and symbiotic Cu nutrition
- *R. irregularis* mainly uses a metal efflux strategy to cope with metal toxicity

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Author statement

Tamara Gómez-Gallego: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft & editing. **M^a Jesús Molina-Luzón:** Methodology & Investigation. **Genevieve Conéjero:** Methodology, Supervision, Writing – review & editing. **Pierre Berthomieu:** Methodology, Supervision, Writing – review & editing. **Nuria Ferrol:** Conceptualization, Methodology, Supervision, Funding acquisition, Project administration, Writing – original draft & editing.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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