



**HAL**  
open science

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

Tamara Gómez-Gallego, Ma Jesús Molina-Luzón, Genevieve Conéjéro, Pierre Berthomieu, Nuria Ferrol

## ► To cite this version:

Tamara Gómez-Gallego, Ma Jesús Molina-Luzón, Genevieve Conéjéro, Pierre Berthomieu, Nuria Ferrol. The arbuscular mycorrhizal fungus *Rhizophagus irregularis* uses the copper exporting ATPase RiCRD1 as a major strategy for copper detoxification. *Environmental Pollution*, 2024, 341, pp.122990. 10.1016/j.envpol.2023.122990 . hal-04311415

**HAL Id: hal-04311415**

**<https://hal.inrae.fr/hal-04311415>**

Submitted on 28 Nov 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Journal Pre-proof



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

Tamara Gómez-Gallego, M<sup>a</sup> Jesús Molina-Luzón, Genevieve Conéjéro, Pierre Berthomieu, Nuria Ferrol

PII: S0269-7491(23)01992-9

DOI: <https://doi.org/10.1016/j.envpol.2023.122990>

Reference: ENPO 122990

To appear in: *Environmental Pollution*

Received Date: 25 May 2023

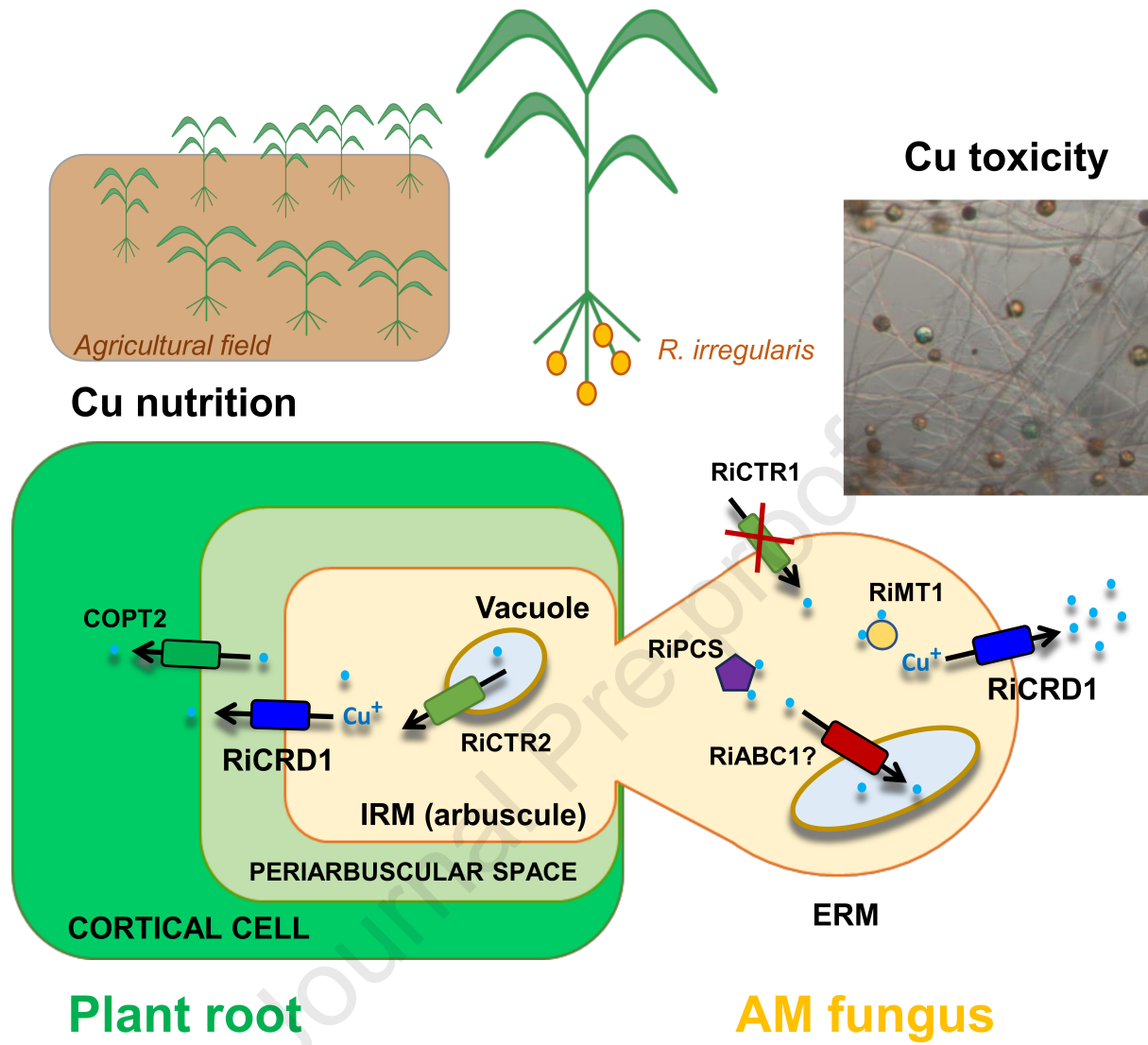
Revised Date: 10 November 2023

Accepted Date: 16 November 2023

Please cite this article as: Gómez-Gallego, T., Molina-Luzón, Ma.Jesú., Conéjéro, G., Berthomieu, P., Ferrol, N., The arbuscular mycorrhizal fungus *Rhizophagus irregularis* uses the copper exporting ATPase RiCRD1 as a major strategy for copper detoxification, *Environmental Pollution* (2023), doi: <https://doi.org/10.1016/j.envpol.2023.122990>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2023 Published by Elsevier Ltd.



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

Tamara Gómez-Gallego<sup>a</sup>, M<sup>a</sup> Jesús Molina-Luzón<sup>a</sup>, Genevieve Conéjéro<sup>b</sup>, Pierre Berthomieu<sup>b</sup> and Nuria Ferrol<sup>a\*</sup>

<sup>a</sup> *Soil and Plant Microbiology Department, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain*

<sup>b</sup> *Institut des Sciences des Plantes de Montpellier, Université de Montpellier, Centre National de la Recherche Scientifique, Institut Agro Montpellier, Institut National de Recherche pour l'Agriculture l'Alimentation et l'Environnement, Montpellier, France*

\*Corresponding author:

E-mail address: nuria.ferrol@eez.csic.es (N. Ferrol)

## 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<sup>+</sup> efflux protein of the P<sub>1B1</sub>-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 ( $\text{Cu}^{2+}$ ) and a  
35 reduced ( $\text{Cu}^+$ ) 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<sub>1B</sub>-  
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<sub>1B</sub>-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<sub>1B</sub>-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  $\mu\text{M}$   $\text{CuSO}_4$ ) or with M-C medium supplemented with 250  $\mu\text{M}$   $\text{CuSO}_4$ , 500  
136  $\mu\text{M}$   $\text{CuSO}_4$  or 45  $\mu\text{M}$   $\text{CdSO}_4$ . 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× modified Hoagland nutrient solution containing 125 µM KH<sub>2</sub>PO<sub>4</sub> and 0.16 µM  
161 CuSO<sub>4</sub> (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 µmol photons m<sup>-2</sup> s<sup>-1</sup>. Roots  
172 were harvested 8 weeks after inoculation.

173 The *Saccharomyces cerevisiae* strains used in this study were the mutants  
174 DTY113 (*cup1Δ*) and WYT (*yap1Δ*), 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 $\alpha$  (*RiEF1 $\alpha$* ; GenBank  
184 Accession No. DQ282611), using as internal control the elongation factor 1 $\alpha$  of the  
185 corresponding host plant (*D. carota* *DcEF1 $\alpha$* , GenBank Accession No.  
186 XM\_017391845; *C. intybus* *CiEF1 $\alpha$* , 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  $\mu$ g of each RNA was used for the cDNA  
195 synthesis in a 20  $\mu$ L final volume reaction containing 200 U of SuperScript III Reverse  
196 Transcriptase (Invitrogen) and 2.5  $\mu$ 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 OD<sub>600</sub> 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 CuSO<sub>4</sub> or with 100 μM CdSO<sub>4</sub> and *yap1Δ* cells onto SD medium without uracil  
246 supplemented or not with 2 mM CuSO<sub>4</sub> or with 100 μM CdSO<sub>4</sub>. 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 iQ<sup>TM</sup>5 Multicolor Real-Time PCR  
251 Detection System (Bio-Rad) using iQ<sup>TM</sup> 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 $\alpha$  (*RiEF1 $\alpha$* ; 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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<sup>-1</sup>).  
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 CaCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>), 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  $\mu$ g mL<sup>-1</sup> 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<sub>1B</sub>-type (CP<sub>X</sub>-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 CPCX<sub>6</sub>P motif in the sixth transmembrane helix typical  
325 of the P<sub>1B-1</sub> subgroup of metal ATPases that transport Cu<sup>+</sup> 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 YNX<sub>4</sub>P 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<sup>+</sup> 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 *Claroideoglomerus 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  $\mu\text{M}$  and 2 mM of  $\text{CuSO}_4$ , 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  $\text{CdSO}_4$  concentrations  
380 up to 100  $\mu\text{M}$  (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  $\mu\text{M}$ ) 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  $\mu\text{M}$   $\text{CdSO}_4$  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  $\mu\text{M}$   $\text{CuSO}_4$  (Fig. 6A). In contrast, ERM exposure to 45  $\mu\text{M}$  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  $\mu\text{M}$   $\text{CuSO}_4$  (2-fold increase) and 6 h after ERM exposure to 45  $\mu\text{M}$  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  $\mu\text{M}$  Cu in monoxenic cultures and 0.16  $\mu\text{M}$  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<sub>1B1</sub>-type ATPase CaCRD1 of *C. albicans*

471 (Riggle and Kumamoto, 2000; Weissman *et al.*, 2000). RiCRD1 has all the  
472 characteristic features of P<sub>1B</sub>-type ATPases, and more specifically of those belonging to  
473 the P<sub>1B-1</sub> subgroup, including the complete cd2094 signature of Cu-like proteins that  
474 transport Cu<sup>+</sup> ions and the invariant CPCX<sub>6</sub>P motif typical of the P<sub>1B-1</sub> 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<sub>1B-1</sub> subgroup are  
495 homologous to a number of metal chaperone proteins, can bind Cu<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>



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<sub>1B</sub>-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 Cd<sup>2+</sup> ions, which is  
543 consistent with the fact that the P<sub>1B-1</sub> subgroup of P<sub>1B</sub>-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 *CRDI*-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 *CRDI*-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<sup>+</sup> 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<sup>a</sup> 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.

**627 Funding sources**

628 This work was supported by grant PID2021-1255210B-I00 funded by MCIN/AEI/  
629 10.13039/501100011033 and by “ERDF A way of making Europe”, by the “European  
630 Union”.

**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.

**637 Acknowledgments**

638 We acknowledge the imaging facility MRI, member of the France-BioImaging national  
639 infrastructure supported by the French National Research Agency (ANR-10-INBS-04,  
640 «Investments for the future»), in Montpellier.

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

649 **References**

650 Adle, D.J., Sinani, D., Kim, H., Lee, J., 2007. A Cadmium-transporting P<sub>1B</sub>-type  
651 ATPase in yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 282, 947–955.  
652 <https://doi.org/10.1074/jbc.M609535200>

653 An, J., Zeng, T., Ji, C., de Graaf, S., Zheng, Z., Xiao, T.T., Deng, X., Xiao, S.,  
654 Bisseling, T. Limpens, E., Pan, Z., 2019. A *Medicago truncatula* SWEET  
655 transporter implicated in arbuscule maintenance during arbuscular mycorrhizal  
656 symbiosis. New Phytol. 224, 396-408. <https://doi.org/10.1111/nph.15975>

657 Antsoategi-Uskola, M., Markina-Iñarrairaegui, A., Ugalde, U., 2017. Copper resistance  
658 in *Aspergillus nidulans* relies on the P(I)-Type ATPase CrpA, regulated by the  
659 transcription factor AceA. Front. Microbiol. 8, 912.  
660 <https://doi.org/10.3389/fmicb.2017.00912>

661 Antsoategi-Uskola M., Markina-Iñarrairaegui A., Ugalde U., 2020. New insights into  
662 copper homeostasis in filamentous fungi. Int. Microbiol. 23, 65-73.  
663 <https://doi.org/10.1007/s10123-019-00081-5>

664 Arguello, J.M., Eren, E., Gonzalez-Guerrero, M., 2007. The structure and function of

- 665 heavy metal transport P1B-ATPases. *BioMetals* 20, 233–248.  
666 <https://doi.org/10.1007/s10534-006-9055-6>
- 667 Bååth, E., 1989. Effects of heavy metals in soil on microbial processes and populations  
668 (a review). *Water Air Soil Pollut.* 47, 335–379. <https://doi.org/10.1007/bf00279331>
- 669 Benabdellah, K., Azcón-Aguilar, C., Valderas, A., Speziga, D., Fitzpatrick, TB., Ferrol  
670 N., 2009. *GintPDX1* encodes a protein involved in vitamin B6 biosynthesis that is  
671 up-regulated by oxidative stress in the arbuscular mycorrhizal fungus *Glomus*  
672 *intraradices*. *New Phytol.* 184, 682-693. [https://doi.org/10.1111/j.1469-](https://doi.org/10.1111/j.1469-8137.2009.02978.x)  
673 [8137.2009.02978.x](https://doi.org/10.1111/j.1469-8137.2009.02978.x)
- 674 Benes, V., Leonhardt, T., Sacky, J., Kotrba, P., 2018. Two P1B-1-ATPases of *Amanita*  
675 *strobiliformis* with distinct properties in Cu/Ag transport. *Front. Microbiol.* 9, 747.  
676 <https://doi.org/10.3389/fmicb.2018.00747>
- 677 Bissig, K.D., Wunderli-Ye, H., Duda, P.W., Solioz, M., 2001. Structure-function  
678 analysis of purified *Enterococcus hirae* CopB copper ATPase: effect of  
679 Menkes/Wilson disease mutation homologues. *Biochem. J.* 357, 217–223.  
680 <https://doi.org/10.1042/bj3570217>
- 681 Bolchi, A., Ruotolo, R., Marchini, G., Vurro, E., di Toppi, L.S., Kohler, A., Tisserant,  
682 E., Martin, F., Ottonello, S., 2011. Genome-wide inventory of metal homeostasis-  
683 related gene products including a functional phytochelatin synthase in the  
684 hypogeous mycorrhizal fungus *Tuber melanosporum*. *Fungal Genet. Biol.* 48, 573–  
685 584. <https://doi.org/10.1016/j.fgb.2010.11.003>
- 686 Brands, M., Dörmann, P., 2022. Two AMP-binding domain proteins from *Rhizophagus*  
687 *irregularis* involved in import of exogenous fatty acids. *Mol. Plant Microbe*.  
688 *Interact.* 35, 464-476. <https://doi.org/10.1094/MPMI-01-22-0026-R>



- 689 Brundrett, M.C., Tedersoo, L., 2018. Evolutionary history of mycorrhizal symbioses  
690 and global host plant diversity. *New Phytol.* 220, 1108–1115.  
691 <https://doi.org/10.1111/nph.14976>
- 692 Camakaris, J., Voskoboinik, I., Mercer, J.F., 1999. Molecular mechanisms of copper  
693 homeostasis. *Biochem. Biophys. Res. Commun.* 261, 225–232.  
694 <https://doi.org/10.1006/bbrc.1999.1073>
- 695 Chabot, S., Bécard, G., Piché, Y., 1992. Life cycle of *Glomus intraradix* in root organ  
696 culture. *Mycologia* 84, 315–321. <https://doi.org/10.2307/3760183>
- 697 Chen, E. C. H, Morin, E., Beaudet, D., Noel, J., Yildirim, G., Ndikumana, S., *et al.* 2018.  
698 High intraspecific genome diversity in the model arbuscular mycorrhizal symbiont  
699 *Rhizophagus irregularis*. *New Phytol.* 220, 1161–1171.  
700 <https://doi.org/10.1111/nph.14989>
- 701 Cobbett, C.S., 2000a. Phytochelatins and their roles in heavy metal detoxification. *Plant*  
702 *Physiol.* 123, 825–832. <https://doi.org/10.1104/pp.123.3.825>
- 703 Cobbett, C.S., 2000b. Phytochelatin biosynthesis and function in heavy-metal  
704 detoxification. *Curr. Opin. Plant Biol.* 3, 211–216. [https://doi.org/10.1016/S1369-](https://doi.org/10.1016/S1369-5266(00)80067-9)  
705 [5266\(00\)80067-9](https://doi.org/10.1016/S1369-5266(00)80067-9)
- 706 Coccina, A., Cavagnaro, T.R., Pellegrino, E., Ercoli, L., McLaughlin, M.J., Watts-  
707 Williams S.J., 2019. The mycorrhizal pathway of zinc uptake contributes to zinc  
708 accumulation in barley and wheat grain. *BMC Plant Biol.* 19, 133.  
709 <https://doi.org/10.1186/s12870-019-1741-y>
- 710 Cornejo, P., Pérez-Tienda, J., Meier, S., Valderas, A., Borie, F., Azcón-Aguilar, C.,  
711 Ferrol, N., 2013. Copper compartmentalization in spores as a survival strategy of

- 712 arbuscular mycorrhizal fungi in Cu-polluted environments. *Soil Biol. Biochem.* 57,  
713 925–928. <https://doi.org/10.1016/j.soilbio.2012.10.031>
- 714 Ecker, D.J., Butt, T.R., Sternberg, E.J., Neeper, M.P., Debouck, C., Gorman, J.A.,  
715 Crooke, S.T., 1986. Yeast metallothionein function in metal ion detoxification. *J.*  
716 *Biol. Chem.* 261, 16895–16900. [https://doi.org/10.1016/S0021-9258\(19\)75973-0](https://doi.org/10.1016/S0021-9258(19)75973-0)
- 717 Ezawa, T., Maruyama, H., Kikuchi, Y., Yokoyama, K., Masuta, C., 2020. Application  
718 of virus-induced gene silencing to arbuscular mycorrhizal fungi, in: Ferrol, N.,  
719 Lanfranco, L. (Eds.), *Arbuscular mycorrhizal fungi: methods and protocols.*  
720 *Methods Mole. Biol.* 2146, 249-254
- 721 Ferrol, N., Azcón-Aguilar, C., Pérez-Tienda, J., 2019. Review: Arbuscular mycorrhizas  
722 as key players in sustainable plant phosphorus acquisition: An overview on the  
723 mechanisms involved. *Plant Sci.* 280, 441-447.  
724 <https://doi.org/10.1016/j.plantsci.2018.11.011>
- 725 Ferrol, N., González-Guerrero, M., Valderas, A., Benabdellah, K., Azcón-Aguilar, C.,  
726 2009. Survival strategies of arbuscular mycorrhizal fungi in Cu-polluted  
727 environments. *Phytochem. Rev.* 8, 551–559. [https://doi.org/10.1007/s11101-009-](https://doi.org/10.1007/s11101-009-9133-9)  
728 9133-9
- 729 Ferrol, N., Tamayo, E., Vargas, P., 2016. The heavy metal paradox in arbuscular  
730 mycorrhizas: from mechanisms to biotechnological applications. *J. Exp. Bot.* 67,  
731 6253–6265. <https://doi.org/10.1093/jxb/erw403>
- 732 Festa, R.A., Thiele, D.J., 2011. Copper: an essential metal in biology. *Curr. Biol.* 21,  
733 R877-R883. <https://doi.org/10.1016/j.cub.2011.09.040>.
- 734 Garcia K., Haider M.Z., Delteil A., Corratgé-Faillie C., Conéjero G., Tattry M., Becquer

- 735 A., Amenc L., Sentenac H., Plassard P., Zimmermann S., 2013. Promoter-  
736 dependent expression of the fungal transporter HcPT1. 1 under Pi shortage and its  
737 spatial localization in ectomycorrhiza. *Fungal Genet. Biol.* 58, 53-61.  
738 <https://doi.org/10.1016/j.fgb.2013.06.007>
- 739 Gietz, R. D., and Schiestl, R. H., 2007. High-efficiency yeast transformation using the  
740 LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* 2, 31–34.  
741 <https://doi.org/10.1038/nprot.2007.13>
- 742 Gómez-Gallego, T., Benabdellah, K., Merlos, M.A., Jiménez-Jiménez, A.M., Alcon, C.,  
743 Berthomieu, P., Ferrol, N., 2019. The *Rhizophagus irregularis* genome encodes  
744 two CTR copper transporters that mediate Cu import into the cytosol and a CTR-  
745 like protein likely involved in copper tolerance. *Front. Plant Sci.* 10. 604  
746 <https://doi.org/10.3389/fpls.2019.00604>
- 747 Gómez-Gallego, T., Valderas, A., van Tuinen, D., Ferrol, N., 2022. Impact of  
748 arbuscular mycorrhiza on maize P(1B)-ATPases gene expression and ionome in  
749 copper-contaminated soils. *Ecotoxicol. Env. Saf.* 234, 113390.  
750 <https://doi.org/10.1016/j.ecoenv.2022.113390>
- 751 González-Guerrero, M., Benabdellah, K., Valderas, A., Azcón-Aguilar, C., Ferrol, N.,  
752 2010. *GintABC1* encodes a putative ABC transporter of the MRP subfamily  
753 induced by Cu, Cd, and oxidative stress in *Glomus intraradices*. *Mycorrhiza* 20,  
754 137–146. <https://doi.org/10.1007/s00572-009-0273-y>
- 755 González-Guerrero, M., Cano, C., Azcón-Aguilar, C., Ferrol, N., 2007. *GintMT1*  
756 encodes a functional metallothionein in *Glomus intraradices* that responds to  
757 oxidative stress. *Mycorrhiza* 17, 327–335. [https://doi.org/10.1007/s00572-007-](https://doi.org/10.1007/s00572-007-0108-7)  
758 0108-7

- 759 González-Guerrero, M., Melville, L.H., Ferrol, N., Lott, J.N.A., Azcón-Aguilar, C.,  
760 Peterson, R.L., 2008. Ultrastructural localization of heavy metals in the  
761 extraradical mycelium and spores of the arbuscular mycorrhizal fungus *Glomus*  
762 *intraradices*. *Can. J. Microbiol.* 54, 103–110. <https://doi.org/10.1139/w07-119>
- 763 Halliwell, B., Gutteridge, J.M., 1984. Oxygen toxicity, oxygen radicals, transition  
764 metals and disease. *Biochem. J.* 219, 1–14. <https://doi.org/10.1042/bj2190001>
- 765 Hamer, D.H., 1986. Metallothionein. *Annu. Rev. Biochem.* 55, 913-951.  
766 <https://doi.org/10.1146/annurev.bi.55.070186.004405>
- 767 Hartmann, M., Voß, S., Requena, N., 2020. Host-induced gene silencing of arbuscular  
768 mycorrhizal fungal genes via *Agrobacterium rhizogenes*-mediated root  
769 transformation in *Medicago truncatula*, in: Ferrol, N., Lanfranco, L. (Eds.),  
770 Arbuscular mycorrhizal fungi: methods and protocols. *Methods Mole. Biol.* 2146,  
771 239–249
- 772 Heiss, S., Wachter, A., Bogs, J., Cobbett, C., Rausch, T., 2003. Phytochelatin synthase  
773 (PCS) protein is induced in *Brassica juncea* leaves after prolonged Cd exposure. *J.*  
774 *Exp. Bot.* 54, 1833–1839. <https://doi.org/10.1093/jxb/erg205>
- 775 Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., Requena, N., 2011. A  
776 versatile monosaccharide transporter that operates in the arbuscular mycorrhizal  
777 fungus *Glomus* sp is crucial for the symbiotic relationship with plants. *Plant Cell*  
778 23, 3812–3823. <https://doi.org/10.1105/tpc.111.089813>
- 779 Hui, J., An, X., Li, Z., Neuhäuser, B., Ludewig, U., Wu, X., Schulze, W.X., Chen, F.,  
780 Feng, G., Lambers, H., Zhang, F., Yuan, L., 2022. The mycorrhiza-specific  
781 ammonium transporter ZmAMT3;1 mediates mycorrhiza-dependent nitrogen  
782 uptake in maize roots. *Plant Cell.* 34, 4066-4087.

- 783 <https://doi.org/10.1093/plcell/koac225>
- 784 Jabnounge, M., Espeout, S., Mieulet, D., Fizames, C., Verdeil, J.L., Conejero, G.,  
785 Rodriguez-Navarro, A., Sentenac, H., Guiderdoni, E., Abdelly, C., Very, A.A.,  
786 2009. Diversity in expression patterns and functional properties in the rice HKT  
787 transporter family. *Plant Physiol.* 150, 1955–1971.  
788 <https://doi.org/10.1104/pp.109.138008>
- 789 Jiang, Y., Wang, W., Xie, Q., Liu, N., Liu, L., Wang, D., Zhang, X., Yang, C., Chen,  
790 X., Tang, D., Wang, E., 2017. Plants transfer lipids to sustain colonization by  
791 mutualistic mycorrhizal and parasitic fungi. *Science* 356, 1172-1175.  
792 <https://doi.org/10.1126/science.aam9970>
- 793 Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., Von Haeseler, A., Jermin, L.S.,  
794 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat.*  
795 *Methods* 14, 587-589. <https://doi.org/10.1038/nmeth.4285>
- 796 Kay, R., Chan, A., Daly, M., McPherson, J., 1987. Duplication of CaMV 35S promoter  
797 sequences creates a strong enhancer for plant genes. *Science* 236, 1299–1302.  
798 <https://doi.org/10.1126/science.236.4806.1299>
- 799 Kobayashi, Y., Maeda, T., Yamaguchi, K., Kameoka, H., Tanaka, S., Ezawa, T.,  
800 Shigenobu, S., Kawaguchi, M., 2018. The genome of *Rhizophagus clarus* HR1  
801 reveals a common genetic basis for auxotrophy among arbuscular mycorrhizal  
802 fungi. *BMC Genomics* 19, 465. <https://doi.org/10.1186/s12864-018-4853-0>
- 803 Kuge, S., and Jones, N., 1994. YAP1 dependent activation of *TRX2* is essential for the  
804 response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides.  
805 *EMBO J.* 13, 655–664. <https://doi.org/10.1002/j.1460-2075.1994.tb06304.x>

- 806 Lanfranco, L., Fiorilli, V., Gutjahr, C., 2018. Partner communication and role of  
807 nutrients in the arbuscular mycorrhizal symbiosis. *New Phytol.* 220, 1031-1046.  
808 <https://doi.org/10.1111/nph.15230>
- 809 Lee, Y.-J., George, E., 2005. Contribution of mycorrhizal hyphae to the uptake of metal  
810 cations by cucumber plants at two levels of phosphorus supply. *Plant Soil* 278,  
811 361–370. <https://doi.org/10.1007/s11104-005-0373-1>
- 812 Letunic, I., Bork, P., 2016. Interactive tree of life (iTOL) v3: an online tool for the  
813 display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44,  
814 W242-245. <https://doi.org/10.1093/NAR/GKW290>
- 815 Li, X.-L., Marschner, H., George, E., 1991. Acquisition of phosphorus and copper by  
816 VA-mycorrhizal hyphae and root-to-shoot transport in white clover. *Plant Soil* 136,  
817 49–57. <https://doi.org/10.1007/bf02465219>
- 818 Linder, M. C., 1991. *Biochemistry of Copper*, Plenum Press, New York
- 819 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using  
820 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25,  
821 402–408. <https://doi.org/10.1006/meth.2001.1262>
- 822 Luginbuehl, L.H., Oldroyd, G.E.D., 2017. Understanding the arbuscule at the heart of  
823 endomycorrhizal symbioses in plants. *Curr. Biol.* 27, R952-963.  
824 <https://doi.org/10.1016/j.cub.2017.06.042>
- 825 Ma Y., Ankit, Tiwari J., Baudhh K., 2022. Plant-mycorrhizal fungi interactions in  
826 phytoremediation of geogenic contaminated soils. *Front. Microbiol.* 13, 843415.  
827 <https://doi.org/10.3389/fmicb.2022.843415>.
- 828 MacLean, A.M., Bravo, A., Harrison, M.J., 2017. Plant signaling and metabolic

- 829 pathways enabling arbuscular mycorrhizal symbiosis. *Plant Cell* 29, 2319–2335.  
830 <https://doi.org/10.1105/tpc.17.00555>
- 831 Macomber, L., Imlay, J.A., 2009. The iron-sulfur clusters of dehydratases are primary  
832 intracellular targets of copper toxicity. *Proc. Natl. Acad. Sci. U. S. A.* 106, 8344–  
833 8349. <https://doi.org/10.1073/pnas.0812808106>
- 834 Malar C, M., Krüger, M., Krüger, C., Wang, Y., Stajich, J.E., Keller, J., Chen, E.C.H.,  
835 Yildirim, G., Villeneuve-Laroche, M., Roux, C., Delaux, P.M., Corradi, N., 2021.  
836 The genome of *Geosiphon pyriformis* reveals ancestral traits linked to the  
837 emergence of the arbuscular mycorrhizal symbiosis. *Curr. Biol.* 31. 1570-1577.  
838 <https://doi.org/10.1016/j.cub.2021.01.058>
- 839 Mendoza-Cozatl, D.G., Zhai, Z., Jobe, T.O., Akmakjian, G.Z., Song, W.Y., Limbo, O.,  
840 Russell, M.R., Kozlovskyy, V.I., Martinoia, E., Vatamaniuk, O.K., Russell, P.,  
841 Schroeder, J.I., 2010. Tonoplast-localized Abc2 transporter mediates phytochelatin  
842 accumulation in vacuoles and confers cadmium tolerance. *J. Biol. Chem.* 285,  
843 40416–40426. <https://doi.org/10.1074/jbc.M110.155408>
- 844 Migocka, M., 2015. Copper-transporting ATPases: The evolutionarily conserved  
845 machineries for balancing copper in living systems. *IUBMB Life* 67, 737–745.  
846 <https://doi.org/10.1002/iub.1437>
- 847 Moreno Jiménez, E., Ferrol, N., Corradi, N., Peñalosa, J.M., Rillig, M.C. 2023. The  
848 potential of arbuscular mycorrhizal fungi to enhance metallic micronutrient uptake  
849 and mitigate food contamination in agriculture: prospects and challenges. *New*  
850 *Phytol.* <https://doi.org/10.1111/nph.19269>
- 851 Morin, E., Miyauchi, S., San Clemente, H., Chen, E.C.H., Pelin, A., de la Providencia,  
852 I., Ndikumana, S., Beaudet, D., Hainaut, M., Drula, E., Kuo, A., Tang, N., Roy, S.,

- 853 Viala, J., Henrissat, B., Grigoriev, I. V, Corradi, N., Roux, C., Martin, F.M., 2019.  
854 Comparative genomics of *Rhizophagus irregularis*, *R. cerebriforme*, *R. diaphanus*  
855 and *Gigaspora rosea* highlights specific genetic features in Glomeromycotina.  
856 *New Phytol.* 222, 1584–1598. <https://doi.org/10.1111/nph.15687>
- 857 Nevitt, T., Ohrvik, H., Thiele, DJ., 2012. Charting the travels of copper in eukaryotes  
858 from yeast to mammals. *Biochim. Biophys.* 1823, 1580-1593.  
859 <https://doi.org/10.1016/j.bbamcr.2012.02.011>
- 860 Nguyen, L.T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: A fast  
861 and effective stochastic algorithm for estimating maximum-likelihood phylogenies.  
862 *Mol. Biol. Evol.* 32. 268-274. <https://doi.org/10.1093/molbev/msu300>
- 863 Pal, R., Rai, J.P.N., 2010. Phytochelatins: peptides involved in heavy metal  
864 detoxification. *Appl. Biochem. Biotechnol.* 160, 945–963.  
865 <https://doi.org/10.1007/s12010-009-8565-4>
- 866 Palmgren, M.G., Nissen, P., 2011. P-type ATPases. *Annu. Rev. Biophys.* 40, 243–266.  
867 <https://doi.org/10.1146/annurev.biophys.093008.131331>
- 868 Park, J., Song, WY., Ko, D., Eom, Y., Hansen, TH., Schiller, M., Lee, TG., Martinoia,  
869 E., Lee, Y., 2012. The phytochelatin transporters AtABCC1 and AtABCC2  
870 mediate tolerance to cadmium and mercury. *Plant J.* 69, 278-288.  
871 <https://doi.org/10.1111/j.1365-313X.2011.04789.x>
- 872 Pepe, A., Sbrana, C., Ferrol, N., Giovannetti, M., 2017. An *in vivo* whole-plant  
873 experimental system for the analysis of gene expression in extraradical mycorrhizal  
874 mycelium. *Mycorrhiza* 27, 659–668. <https://doi.org/10.1007/s00572-017-0779-7>
- 875 Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining



- 876 parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of  
877 infection. *Trans. Br. Mycol. Soc.* 55, 158–161. [https://doi.org/10.1016/S0007-](https://doi.org/10.1016/S0007-1536(70)80110-3)  
878 1536(70)80110-3
- 879 Pozo, M.J., Zabalgoceazcoa, I., de Aldana, B.R.V., and Martinez-Medina, A., 2021.  
880 Untapping the potential of plant mycobiomes for applications in agriculture. *Curr.*  
881 *Opin. Plant Biol.* 60, 102034. <https://doi.org/10.1016/j.pbi.2021.102034>
- 882 Rekha, K., Balasundaram, U., Keeran, NS., 2021. 3 - Role of ABC transporters and  
883 other vacuolar transporters during heavy metal stress in plants, in: Roychoudhury,  
884 A., Tripathi, DK., Deshmukh, R. (Eds.), *Metal and nutrient transporters in abiotic*  
885 *stress*. Academic Press, 55-76
- 886 Rensing, C., Fan, B., Sharma, R., Mitra, B., Rosen, BP., 2000. CopA: An *Escherichia*  
887 *coli* Cu(I)-translocating P-type ATPase. *Proc. Natl. Acad. Sci. U S A.* 97, 652-656.  
888 <https://doi.org/10.1073/pnas.97.2.652>
- 889 Rensing, C., Ghosh, M., Rosen, B.P., 1999. Families of soft-metal-ion-transporting  
890 ATPases. *J. Bacteriol.* 181, 5891–5897. [https://doi.org/10.1128/JB.181.19.5891-](https://doi.org/10.1128/JB.181.19.5891-5897.1999)  
891 5897.1999
- 892 Riggle, P.J., Kumamoto, C.A., 2000. Role of a *Candida albicans* P1-type ATPase in  
893 resistance to copper and silver ion toxicity. *J. Bacteriol.* 182, 4899–4905.  
894 <https://doi.org/10.1128/JB.182.17.4899-4905.2000>
- 895 Roth, R., Paszkowski, U., 2017. Plant carbon nourishment of arbuscular mycorrhizal  
896 fungi. *Curr. Opin. Plant Biol.* 39, 50–56. <https://doi.org/10.1016/j.pbi.2017.05.008>
- 897 Ruytinx, J., Kafle, A., Usman, M., Coninx, L., Zimmermann, García, K., 2020.  
898 Micronutrient transport in mycorrhizal symbiosis; zinc steals the show. *Fungal*

- 899 Biol. Rev. 34, 1-9. <https://doi.org/10.1016/j.fbr.2019.09.001>
- 900 Saitoh, Y., Izumitsu, K., Tanaka, C., 2009. Phylogenetic analysis of heavy-metal  
901 ATPases in fungi and characterization of the copper-transporting ATPase of  
902 *Cochliobolus heterostrophus*. Mycol. Res. 113, 737–745.  
903 <https://doi.org/10.1016/j.mycres.2009.02.009>
- 904 Salustros, N., Grønberg, C., Abeyrathna, N.S. *et al.* 2022. Structural basis of ion uptake  
905 in copper-transporting P1B-type ATPases. Nat. Commun. 13, 5121  
906 <https://doi.org/10.1038/s41467-022-32751-w>
- 907 Senovilla, M., Abreu, I., Escudero, V., Cano, C., Bago, A., Imperial, J., González-  
908 Guerrero, M., 2020. MtCOPT2 is a Cu<sup>+</sup> transporter specifically expressed in  
909 *Medicago truncatula* mycorrhizal roots. Mycorrhiza 30, 781–788.  
910 <https://doi.org/10.1007/s00572-020-00987-3>
- 911 Shi, J., Wang, X., Wang, E., 2023. Mycorrhizal symbiosis in plant growth and stress  
912 adaptation: from genes to ecosystems. Annu. Rev. Plant Biol. 74, 569-607.  
913 <https://doi.org/10.1146/annurev-arplant-061722-090342>
- 914 Shi, W., Zhang, Y., Chen, S., Polle, A., Rennenberg, H., Luo, Z-B., 2019. Physiological  
915 and molecular mechanisms of heavy metal accumulation in nonmycorrhizal versus  
916 mycorrhizal plants. Plant Cell Environ. 42, 1087– 1103.  
917 <https://doi.org/10.1111/pce.13471>
- 918 Shine, A.M., Shakya, V.P.S., Idnurm, A., 2015. Phytochelatin synthase is required for  
919 tolerating metal toxicity in a basidiomycete yeast and is a conserved factor  
920 involved in metal homeostasis in fungi. Fungal Biol. Biotechnol. 2, 3.  
921 <https://doi.org/10.1186/s40694-015-0013-3>

- 922 Smith, A.T., Smith, K.P., Rosenzweig, A.C., 2014. Diversity of the metal-transporting  
923 P1B-type ATPases. *J. Biol. Inorg. Chem.* 19, 947–960.  
924 <https://doi.org/10.1007/s00775-014-1129-2>
- 925 Solioz, M., Vulpe, C., 1996. CPx-type ATPases: a class of P-type ATPases that pump  
926 heavy metals. *Trends Biochem. Sci.* 21, 237–241. [https://doi.org/10.1016/S0968-](https://doi.org/10.1016/S0968-0004(96)20016-7)  
927 [0004\(96\)20016-7](https://doi.org/10.1016/S0968-0004(96)20016-7)
- 928 Spatafora, J.W., Chang, Y., Benny, G.L., Lazarus, K., Smith, M.E., Berbee, M.L.,  
929 Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., James, T.Y., O'Donnell, K.,  
930 Roberson, R.W., Taylor, T.N., Uehling, J., Vilgalys, R., White, M.M., Stajich, J.E.,  
931 2016. A phylum-level phylogenetic classification of zygomycete fungi based on  
932 genome-scale data. *Mycologia* 108, 1028–1046. <https://doi.org/10.3852/16-042>
- 933 St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., Fortin, J.A., 1996. Enhanced hyphal  
934 growth and spore production of the arbuscular mycorrhizal fungus *Glomus*  
935 *intraradices* in an *in vitro* system in the absence of host roots. *Mycol. Res.* 100,  
936 328–332. [https://doi.org/10.1016/S0953-7562\(96\)80164-X](https://doi.org/10.1016/S0953-7562(96)80164-X)
- 937 Song, WY., Mendoza-Cózatl, DG., Lee, Y., Schroeder, JI., Ahn, SN., Lee, HS., Wicker,  
938 T., Martinoia, E., 2014. Phytochelatin-metal(loid) transport into vacuoles shows  
939 different substrate preferences in barley and *Arabidopsis*. *Plant Cell Environ.* 37,  
940 1192–1201. <https://doi.org/10.1111/pce.12227>
- 941 Strausak, D., Fontaine, S. La, Hill, J., Firth, S.D., Lockhart, P.J., Mercer, J.F.B., 1999.  
942 The role of GMXCXXC metal binding sites in the copper-induced redistribution of  
943 the Menkes protein. *J. Biol. Chem.* 274, 11170–11177.  
944 <https://doi.org/10.1074/jbc.274.16.11170>
- 945 Tamayo, E., Gómez-Gallego, T., Azcón-Aguilar, C., Ferrol, N., 2014. Genome-wide

- 946 analysis of copper, iron and zinc transporters in the arbuscular mycorrhizal fungus  
947 *Rhizophagus irregularis*. Front. Plant Sci. 5, 547.  
948 <https://doi.org/10.3389/fpls.2014.00547>
- 949 Tamai, K.T., Gralla, E.B., Ellerby, L.M., Valentine, J.S., Thiele, D.J., 1993. Yeast and  
950 mammalian metallothioneins functionally substitute for yeast copper-zinc  
951 superoxide dismutase. Proc. Natl. Acad. Sci. USA 90, 8013-8017.  
952 <https://doi.org/10.1073/pnas.90.17.8013>
- 953 Tanzi, R.E., Petrukhin, K., Chernov, I., Pellequer, J.L., Wasco, W., Ross, B., Romano,  
954 D.M., Parano, E., Pavone, L., Brzustowicz, L.M., *et al.*, 1993. The Wilson disease  
955 gene is a copper transporting ATPase with homology to the Menkes disease gene.  
956 Nat. Genet. 5, 344–350. <https://doi.org/10.1038/ng1293-344>
- 957 Thiele, D.J., 1988. ACE1 regulates expression of the *Saccharomyces cerevisiae*  
958 metallothionein gene. Mol. Cell. Biol. 8, 2745–2752.  
959 <https://doi.org/10.1128/mcb.8.7.2745-2752.1988>
- 960 Toone, W.M., Jones, N., 1999. AP-1 transcription factors in yeast. Curr. Opin. Genet.  
961 Dev. 9, 55-61. [https://doi.org/10.1016/s0959-437x\(99\)80008-2](https://doi.org/10.1016/s0959-437x(99)80008-2)
- 962 Trouvelot, A., Kough, J.L., Gianinazzi-Pearson, V., 1986. Estimation of vesicular  
963 arbuscular mycorrhizal infection levels. Research for methods having a functional  
964 significance. Physiol. Genet. Asp. mycorrhizae = Asp. Physiol. Genet. des  
965 mycorrhizes Proc. 1st Eur. Symp. Mycorrhizae, Dijon, 1-5 July 1985.  
966 <https://doi.org/10.3/JQUERY-ULJS>
- 967 Venice, F., Ghignone, S., Salvioli di Fossalunga, A., Amselem, J., Novero, M., Xianan,  
968 X., Sędziewska Toro, K., Morin, E., Lipzen, A., Grigoriev, I. V., Henrissat, B.,

- 969 Martin, F.M., Bonfante, P., 2020. At the nexus of three kingdoms: the genome of  
970 the mycorrhizal fungus *Gigaspora margarita* provides insights into plant,  
971 endobacterial and fungal interactions. *Environ. Microbiol.* 22, 122-141.  
972 <https://doi.org/10.1111/1462-2920.14827>
- 973 Wang, S., Chen, A., Xie, K., Yang, X., Luo, Z., Chen, J., Zeng, D., Ren, Y., Yang, C.,  
974 Wang, L., Feng, H., López-Arredondo, D.L., Herrera-Estrella, L.R., Xu, G., 2020.  
975 Functional analysis of the OsNPF4.5 nitrate transporter reveals a conserved  
976 mycorrhizal pathway of nitrogen acquisition in plants. *Proc. Natl. Acad. Sci.* 117,  
977 16649–16659. <https://doi.org/10.1073/pnas.2000926117>
- 978 Wang, S., Xie, X., Che, X., Lai, W., Ren, Y., Fan, X., Hu, W., Tang, M., Chen, H.,  
979 2023. Host- and virus-induced gene silencing of HOG1-MAPK cascade genes in  
980 *Rhizophagus irregularis* inhibit arbuscule development and reduce resistance of  
981 plants to drought stress. *Plant Biotechnol. J.* 21, 866-883.  
982 <https://doi.org/10.1111/pbi.14006>
- 983 Weissman, Z., Berdicevsky, I., Cavari, B.-Z., Kornitzer, D., 2000. The high copper  
984 tolerance of *Candida albicans* is mediated by a P-type ATPase. *Proc. Natl. Acad.*  
985 *Sci.* 97, 3520–3525. <https://doi.org/10.1073/pnas.97.7.3520>
- 986 Wiemann, P., Perevitsky, A., Lim, FY., Shadkchan, Y., Knox, BP., Landero Figueora,  
987 JA., Choera, T., Niu, M., Steinberger, AJ., Wüthrich, M., Idol, RA., Klein, BS.,  
988 Dinauer, MC., Huttenlocher, A., Osherov, N., Keller, NP., 2017. *Aspergillus*  
989 *fumigatus* copper export machinery and reactive oxygen intermediate defense  
990 counter host copper-mediated oxidative antimicrobial offense. *Cell Rep.* 19, 1008-  
991 1021. <https://doi.org/10.1016/j.celrep.2017.04.019>
- 992 Wipf, D., Krajinski, F., Courty, P.-E., 2019. Trading on the arbuscular mycorrhiza

- 993 market: from arbuscules to common mycorrhizal networks. *New Phytol.* 223,  
994 1127-1142. <https://doi.org/10.1111/nph.15775>
- 995 Wu, A., Wemmie, J.A., Edgington, N.P., Goebel, M., Guevara, J.L., Moye-Rowley, W.S.,  
996 1993. Yeast bZip proteins mediate pleiotropic drug and metal resistance. *J. Biol.*  
997 *Chem.* 268, 18850-18858
- 998 Xie, X., Lai, W., Che, X., Wang, S., Ren, Y., Hu, W., Chen, H., Tang, M., 2022. A SPX  
999 domain-containing phosphate transporter from *Rhizophagus irregularis* handles  
1000 phosphate homeostasis at symbiotic interface of arbuscular mycorrhizas. *New*  
1001 *Phytol.* 234, 650-671. <https://doi.org/10.1111/nph.17973>
- 1002 Yang, K., Shadkchan, Y., Tannous, J., Landero Figueroa, J.A., Wiemann, P., Osharov,  
1003 N., Wang, S., Keller, N.P., 2018. Contribution of ATPase copper transporters in  
1004 animal but not plant virulence of the crossover pathogen *Aspergillus flavus*.  
1005 *Virulence.* 9, 1273–1286. <https://doi.org/10.1080/21505594.2018.1496774>.
- 1006 Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., Klausner, R.D., 1995. The  
1007 Menkes/Wilson disease gene homologue in yeast provides copper to a  
1008 ceruloplasmin-like oxidase required for iron uptake. *Proc. Natl. Acad. Sci. U. S. A.*  
1009 92, 2632–2636. <https://doi.org/10.1073/pnas.92.7.2632>
- 1010
- 1011

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<sub>1B</sub>-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, *Claroideoglomus*  
 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 CuSO<sub>4</sub>, 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 CuSO<sub>4</sub>, 500 μM CuSO<sub>4</sub> (A) or with 45 μM  
 1053 CdSO<sub>4</sub> (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  $2^{-\Delta\Delta CT}$  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  $\mu$ M CuSO<sub>4</sub>, 500  $\mu$ M  
1066 CuSO<sub>4</sub> or 45  $\mu$ M CdSO<sub>4</sub> 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  $2^{-\Delta\Delta CT}$  method using *RiEF1 $\alpha$*  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.

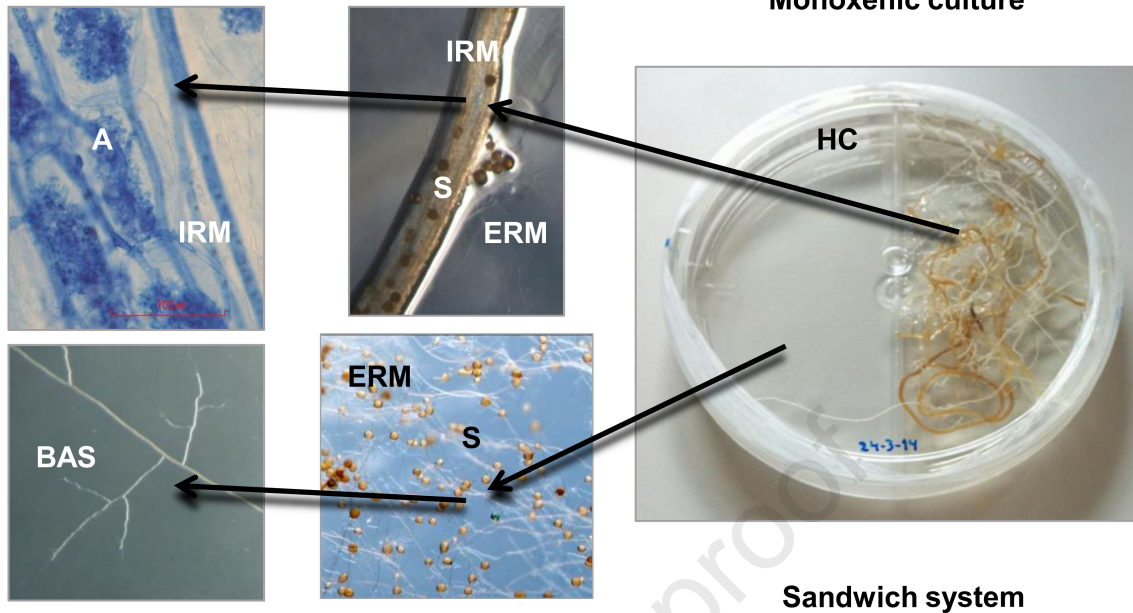
1073 **Fig. 7.** *RiCRD1* transcript levels in the *R. irregularis* ERM and IRM. Transcript levels  
1074 of *RiCRD1* (A) and *RiMST2* (B) were measured in the extraradical mycelia (ERM) and  
1075 the intraradical mycelia (IRM) of *R. irregularis* grown under control conditions in  
1076 monoxenic cultures (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  $2^{-\Delta\Delta CT}$  method  
1079 using *RiEF1 $\alpha$*  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  $\mu\text{m}$ .

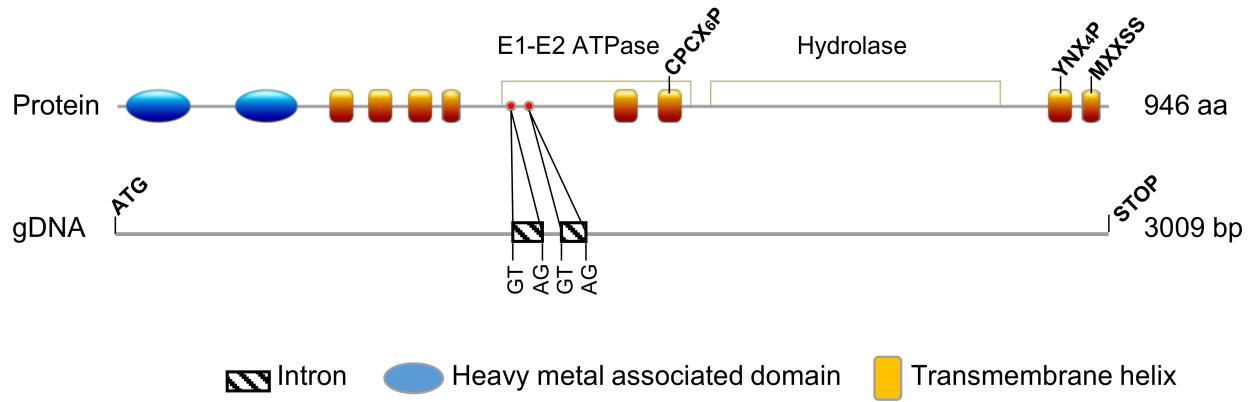
1090 **Fig. 9.** Effect of Cu deficiency on *RiCRDI* transcript levels in the IRM. *R. irregularis*  
1091 colonized roots were grown in presence and in the absence of Cu in two experimental  
1092 systems. Mycorrhizal carrot roots were grown in monoxenic cultures in M media  
1093 (control, 0.5  $\mu\text{M}$  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  $\mu\text{M}$  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 $\alpha$*  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

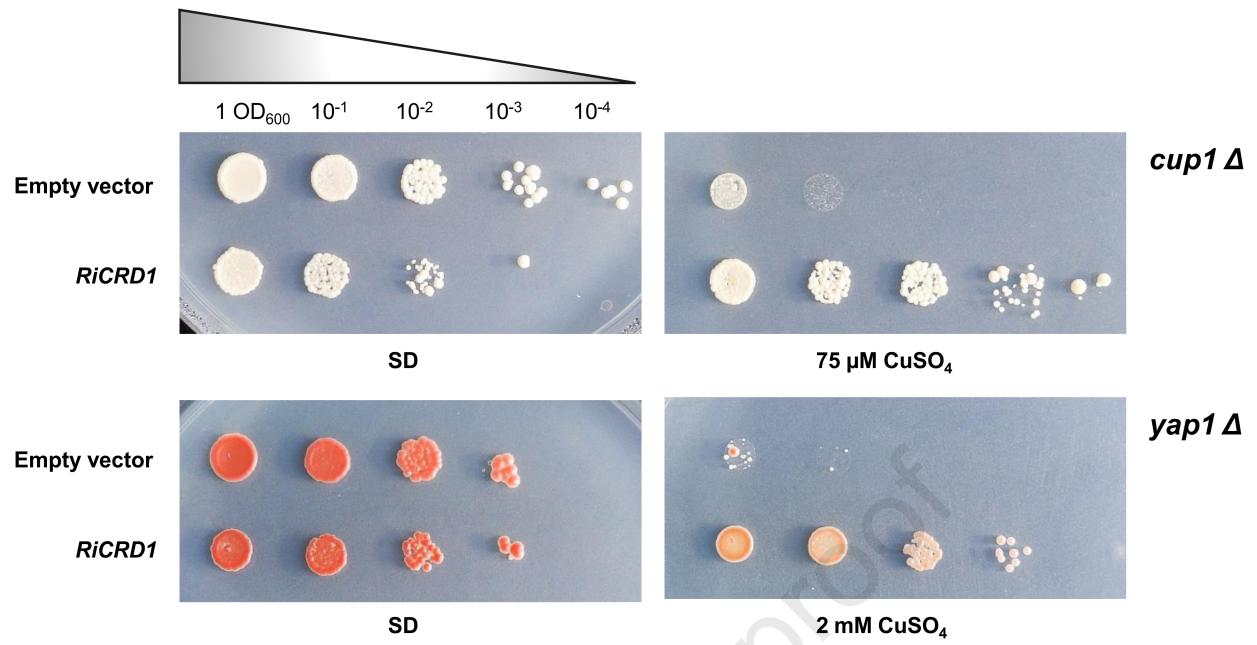


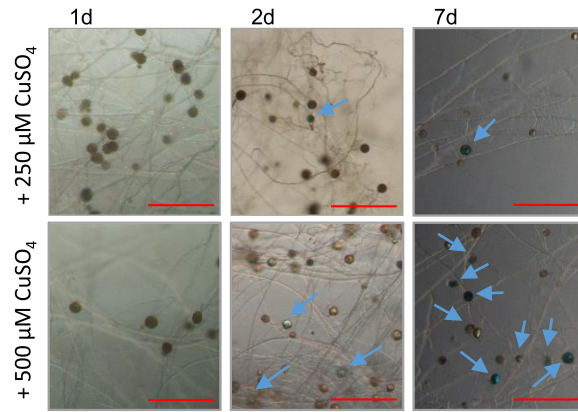
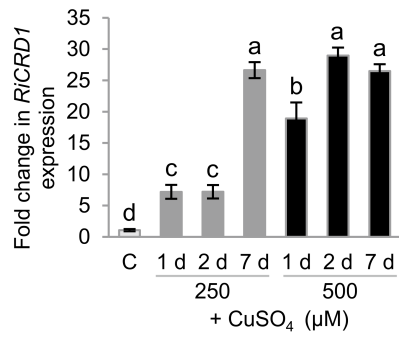
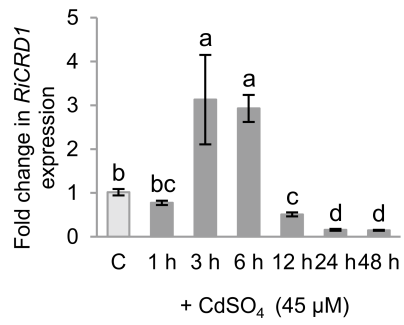
B



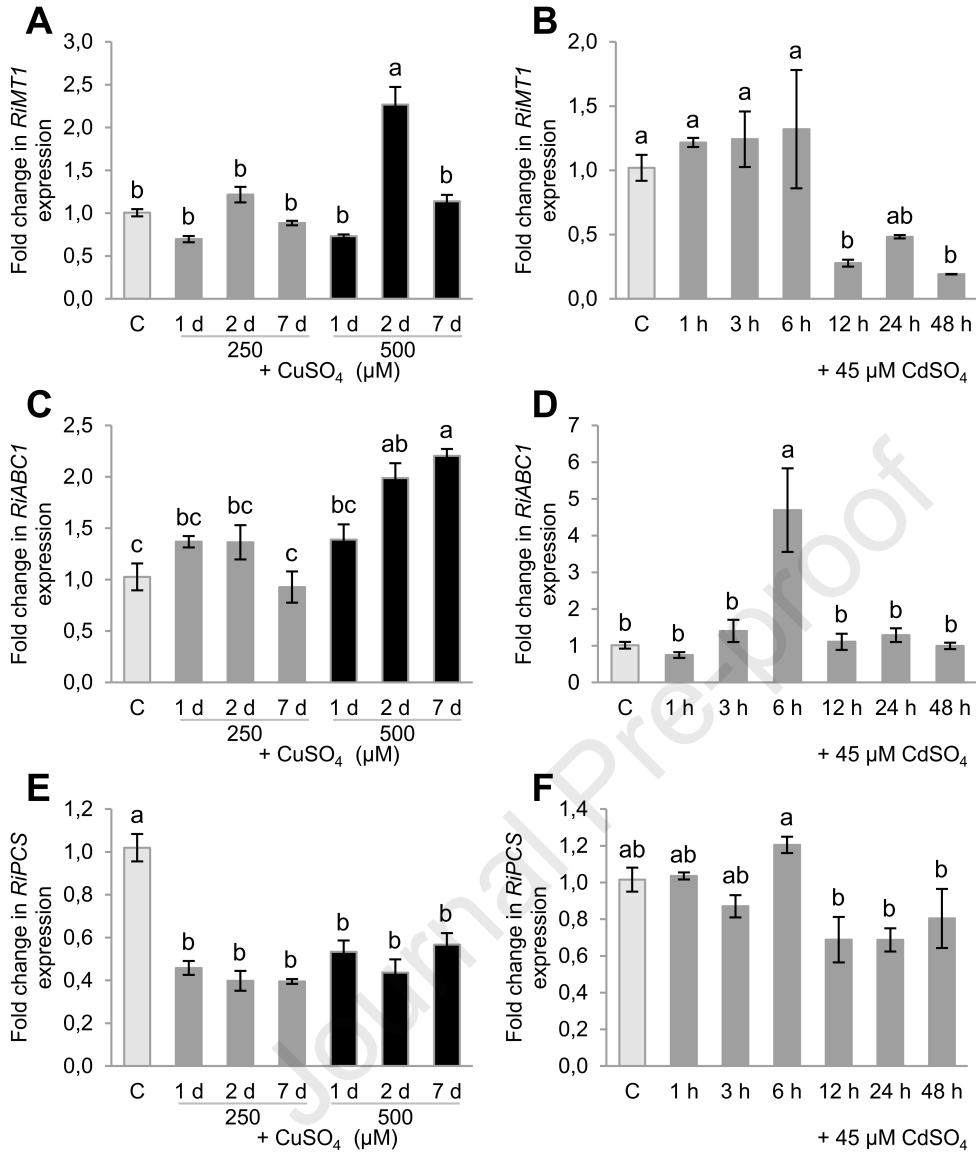




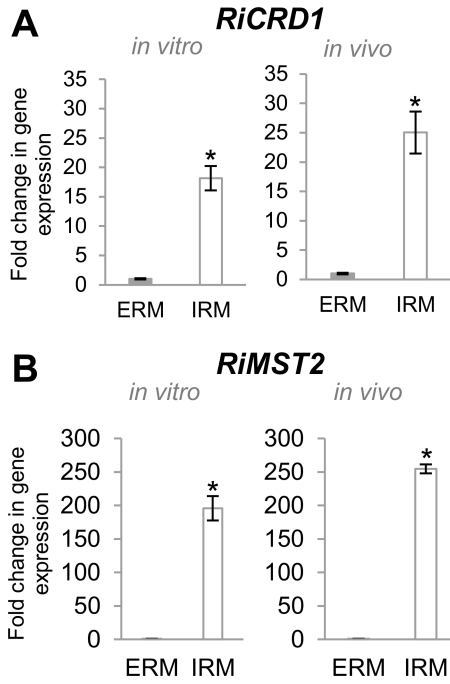


**A****B**

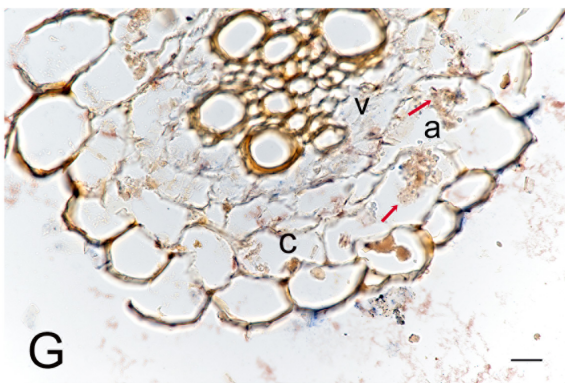
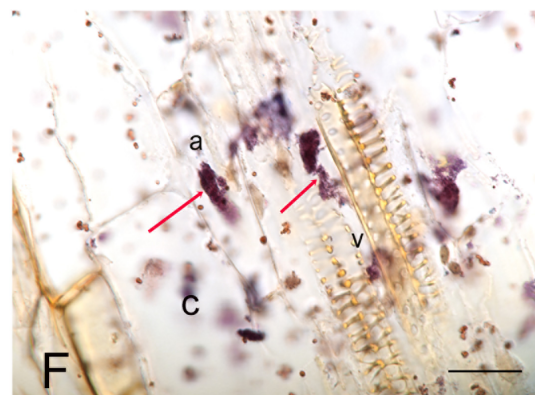
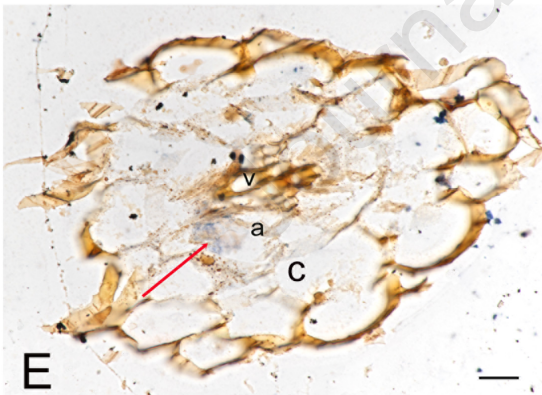
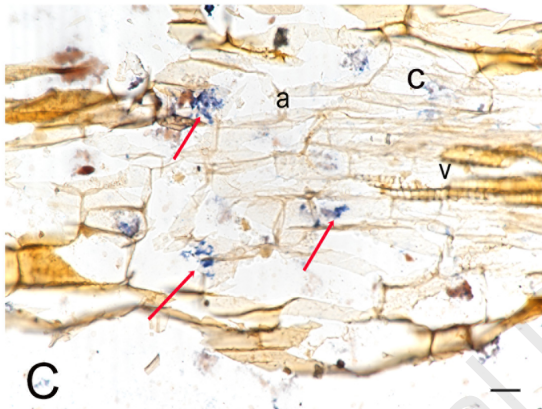
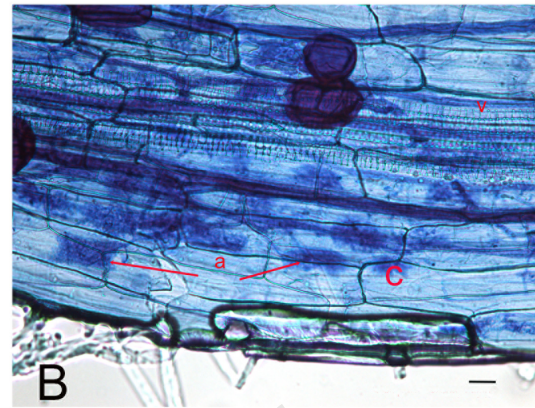
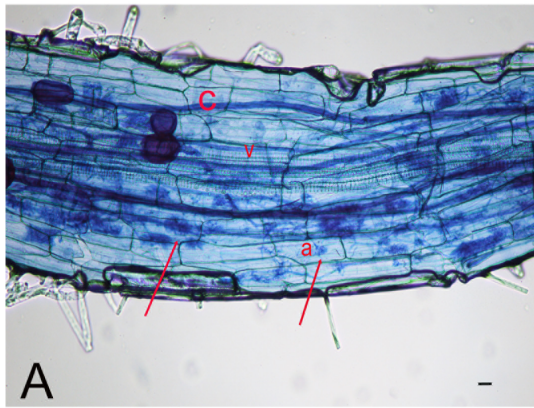
Journal Pre-proof



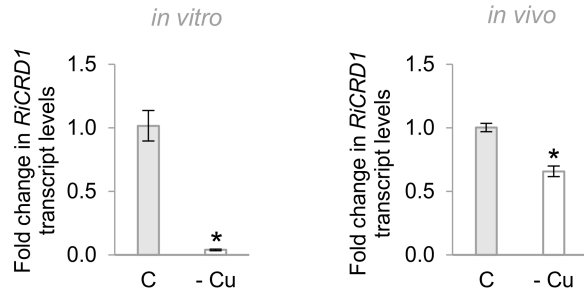




Journal Pre-proof



Journal Pre-proof



Journal Pre-proof

- *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

Journal Pre-proof

**Author statement**

**Tamara Gómez-Gallego:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft & editing. **M<sup>a</sup> 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:

Journal Pre-proof