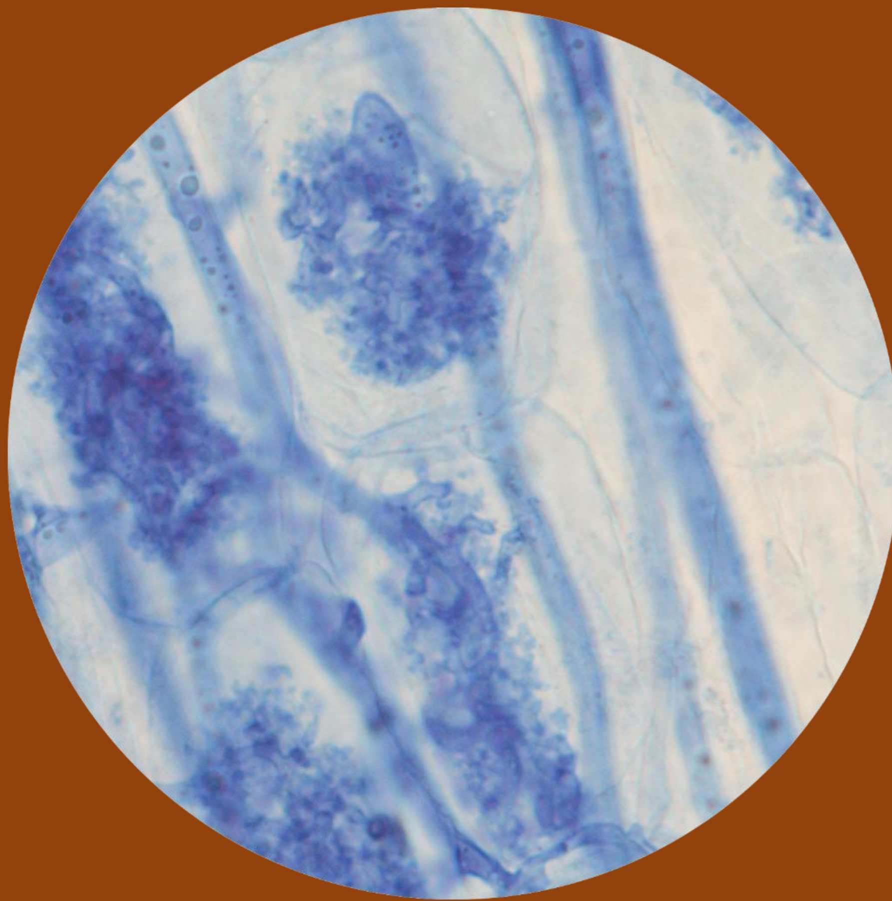
DOCTORAL THESIS

Regulation of Copper Transporters in the Arbuscular Mycorrhizal Symbiosis: Effect on Host Plant Copper Homeostasis and Development



Tamara María Gómez Gallego

Doctoral Program in Fundamental and Systems Biology
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Consejo Superior de Investigaciones Científicas

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Regulation of Copper Transporters in the Arbuscular Mycorrhizal Symbiosis: Effect on Host Plant Copper Homeostasis and Development

Memoria presentada por Dña. Tamara María Gómez Gallego, Licenciada en Biología, para optar al grado de Doctor en Ciencias Biológicas por la Universidad de Granada con Mención Internacional

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ABREVIATURAS

AM: Arbuscular mycorrhiza/l

ANOVA: Análisis de varianza, analisis of variance

BLAST: Basic local alignment search tool

CTR: Copper transporter

Cu: Cobre, copper

ERM: Micelio extraradical, extraradical mycelium

HMA: Heavy metal ATPase

IRM: Micelio intraradical, intraradical mycelium

JGI: Joint Genome Institut

MA: Micorriza/s arbuscular/es o micorrícico arbuscular, en función del contexto

MDS: Mínima diferencia significativa

MEGA: Molecular evolutionary genetics analysis

NCBI: National Center for Biotechnology Information

RACE: Rapid amplification of cDNA ends

SD: Synthetic dextrose

SOD: superóxido dismutasa, superoxide dismutase

YPD: Yeast extract peptone dextrose

Resumen

RESUMEN

El cobre (Cu) es un metal de transición y un micronutriente esencial para el desarrollo de todos los organismos, que actúa como cofactor redox en enzimas clave implicadas en una gran variedad de procesos bioquímicos esenciales para la vida, como la respiración, la eliminación del superóxido, la fotosíntesis, la percepción de etileno y la movilización de hierro. Sin embargo, cuando está en exceso, se vuelve tóxico debido a su capacidad de desplazar otros iones metálicos presentes en centros catalíticos o motivos estructurales de proteínas y a través de la generación de especies de oxígeno reactivo por reacciones tipo Fenton que pueden causar daño oxidativo en ADN, lípidos y proteínas. Debido a la naturaleza dual del Cu, los organismos han desarrollado sofisticadas redes homeostáticas altamente reguladas que les permiten controlar rigurosamente los niveles intracelulares de Cu, en las que transportadores de membrana que median la captación y el eflujo del Cu juegan papeles clave. Aunque el Cu suele estar presente como elemento traza en los suelos, en determinadas áreas, y principalmente como consecuencia de actividades antropogénicas como la minería, la sobrefertilización y el uso de pesticidas, puede alcanzar niveles tóxicos. Por tanto, las plantas, y en general todos los organismos del suelo se enfrentan a una amplia gama de concentraciones ambientales de Cu, que van desde la escasez hasta el exceso.

Una de las estrategias más importantes que han desarrollado las plantas para superar ambas situaciones, es decir, tanto la deficiencia como la toxicidad del Cu, es el establecimiento de una simbiosis mutualista con los hongos micorrícico arbusculares (MA), que son microorganismos del suelo pertenecientes al filo Glomeromycota. El simbionte fúngico, no solo coloniza las células corticales de la raíz, sino que también desarrolla una amplia red de hifas en el suelo que se extiende más allá de la zona de depleción de nutrientes establecida en torno a la raíz, proporcionando a la planta una nueva vía, la vía micorrícica, para la captación y transporte de nutrientes de baja movilidad, como fósforo, nitrógeno, Zn y Cu. Los hongos MA no solo mejoran la nutrición mineral de la planta hospedadora, sino que también incrementan su tolerancia frente a estreses bióticos y abióticos, como salinidad, sequía y contaminación con metales pesados. El potencial de los hongos MA de beneficiar el desarrollo de las plantas tanto en condiciones de deficiencia como de toxicidad de Cu ha sido descrito en numerosos estudios fisiológicos, habiéndose propuesto diferentes mecanismos para explicar el efecto protector de la simbiosis MA frente al estrés causado por metales pesados. Sin embargo, actualmente se desconocen los mecanismos de transporte de Cu en la simbiosis.

En esta tesis doctoral, con el fin de profundizar en el entendimiento de los mecanismos de la homeostasis del Cu en la simbiosis MA, hemos utilizado una combinación de metodologías *in silico*, fisiológicas y moleculares para caracterizar los sistemas de transporte de Cu en la simbiosis. Como modelo experimental hemos utilizado la simbiosis establecida entre el hongo MA modelo *Rhizophagus irregularis*, fácilmente cultivable en cultivo monoxénico y cuyo

genoma se ha secuenciado, y diferentes especies vegetales. Los cultivos de R. *irregularis* se establecieron en dos sistemas experimentales que permiten obtener material fúngico de calidad para los análisis moleculares: un sistema *in vitro* en cultivo monoxénico y otro *in vivo* en un sistema experimental tipo sándwich implementado en la planta hospedadora.

Como primer paso para comprender mejor los mecanismos de la homeostasis de Cu en el simbionte fúngico, se realizó una búsqueda en el genoma de *R. irregularis* de genes que potencialmente codifican proteínas involucradas en el transporte de Cu. Este análisis *in silico* permitió la identificación de siete marcos abiertos de lectura, que potencialmente codifican transportadores de Cu y que pertenecen a dos familias multigénicas. Tres de los genes pertenecien a la familia CTR de transportadores de Cu (*RiCTR1-RiCTR3*) y cuatro a la familia P_{1B}-ATPasa (*RiCCC2.1-.3* y *RiCRD*). La comparación de estas familias génicas con las presentes en otros hongos de referencia reveló una expansión de las ATPasas tipo CCC2 de *R. irregularis*. El análisis de los perfiles transcriptómicos publicados de *R. irregularis* reveló que *RiCTR2* se expresa mayoritariamente en raíces micorrizadas, lo que sugiere que el Cu es importante para la colonización fúngica. Estos resultados se presentan en el **Capítulo I.**

Con el fin de obtener algunos indicios sobre el papel de los CTRs de *R. irregularis* en la simbiosis, llevamos a cabo la primera caracterización funcional de las tres secuencias candidatas previamente identificadas en su genoma (*RiCTR1, RiCTR2* y *RiCTR3*). Estos datos se presentan en el **Capítulo II**. Hemos demostrado que *R. irregularis* expresa dos genes que codifican transportadores de Cu de la familia CTR, *RiCTR1* y *RiCTR2*, y dos variantes de empalme alternativo de un tercer gen, *RiCTR3*. Los análisis funcionales en levadura revelaron que *RiCTR1* codifica un transportador de Cu de membrana plasmática y *RiCTR2* un transportador de Cu vacuolar. *RiCTR1* se expresó mayoritariamente en el micelio extrarradical (ERM) y *RiCTR2* en el micelio intrarradical (IRM). En el ERM, la expresión de *RiCTR1* aumentó bajo condiciones de deficiencia de Cu y se reprimió bajo condiciones de toxicidad. La inducción en la expresión de *RiCTR2* se produjo solamente en el ERM crecido bajo condiciones de deficiencia severa de Cu. Estos datos sugieren que RiCTR1 está involucrado en la adquisición de Cu por el ERM y RiCTR2 en la movilización de las reservas vacuolares de Cu. La deficiencia de Cu disminuyó la colonización micorrícica y la frecuencia de arbúsculos, pero aumentó la expresión de *RiCTR1* y *RiCTR2* en el IRM, lo que sugiere que el IRM tiene una alta demanda de Cu. El tercer gen *RiCTR3* produce dos variantes de empalme alternativo, *RiCTR3A* y *RiCTR3B*, que se expresan mayoritariamente en el ERM. La activación transcripcional de *RiCTR3A* por toxicidad de Cu y su capacidad de revertir la sensibilidad al Cu del mutante *Δyap-1* de levadura sugiere que RiCTR3A podría funcionar como un receptor de Cu que está implicado en la activación de los mecanismos de tolerancia al Cu.

Una vez que demostramos que la captación de Cu por el ERM de *R. irregularis* está mediada por el transportador de membrana de plasma RiCTR1, cuya expresión génica aumenta bajo condiciones de deficiencia de Cu pero disminuye bajo condiciones de la toxicidad de Cu, intentamos identificar el transportador que podría estar involucrado en la transferencia de Cu a la planta en la interfase arbuscular. El eflujo de los iones metálicos tiene lugar a través de P_{1B}-ATPasas, también conocidas como ATPasas de metales pesados (HMA), que acoplan la hidrólisis de ATP al transporte de metales pesados a través de diferentes membranas celulares. El genoma de *R. irregularis* contiene cuatro genes que potencialmente codifican P_{1B}-ATPasas. *RiCCC2.1-3* son ortólogos del gen *CCC2* de *Saccharomyces cerevisaie* que codifica una Cu-ATPasa localizada en el aparato de Golgi que transfiere Cu a cuproproteinas, mientras que *RiCRD1* es el ortólogo de *CaCRD1* de la levadura patógena *Candida albicans*, que codifica una P_{1B}-ATPasa implicada en el eflujo del exceso de Cu fuera de la célula proporcionándole resistencia a este metal. En el **Capítulo III,** con el objetivo de determinar si RiCRD1 podría estar involucrado en el eflujo de Cu al apoplasto de la interfase simbiótica y/o en la tolerancia a metales, se analizaron los patrones de expresión de *RiCRD1* en el IRM y en el ERM de *R. irregularis* crecidos en presencia de diferentes niveles de Cu. Nuestros resultados indican que RiCRD podría ser el transportador responsable de la liberación de Cu al apoplasto en la interfase arbuscular, ya que mediante experimentos de hibridación *in situ* pudimos demostrar la presencia de los transcritos de *RiCRD* en los arbúsculos desarrollados en las células corticales de raíces micorrizadas de tomate. Por otro lado, la expresión de *RiCRD* se indujo por toxicidad de Cu y Cd en el ERM. Estos resultados indican que RiCRD podría tener un papel importante en la detoxificación de Cu y Cd actuando como una bomba de eflujo de metales. La predicción de su localización en la membrana plasmática también apoya esta hipótesis. La mayor inducción en la expresión de *RiCRD* bajo condiciones de toxicidad de Cu que la presentada por otros genes de *R. irregularis* relacionados con la tolerancia a Cu, como el gen *RiMT* que codifica una metalotioneína y *RiABC* un transportador tipo ABC, sugiere que RiCRD es el principal determinante de la tolerancia a Cu en *R. irregularis.* Esta es la primera vez que se describe una estrategia basada en el eflujo de Cu en hongos MA para hacer frente al exceso del metal. En general, los resultados presentados indican que la ATPasa RiCRD podría desempeñar un papel dual, en la detoxificación del exceso de Cu y en la nutrición simbiótica de Cu, siendo, por lo tanto, un transportador clave en la homeostasis de Cu en *R. irregularis.*

Finalmente, hemos analizado el efecto de la micorrización sobre el desarrollo de la planta hospedadora y los mecanismos de transporte implicados en la detoxificación de Cu bajo condiciones de toxicas de este metal. En el **Capítulo IV**, se detalla como la inoculación de un cultivar sensible al Cu de *Zea mays* con el hongo MA *R. irregularis* modifica la respuesta fisiológica de la planta a la toxicidad de Cu y la expresión génica de las HMAs. Las plantas

micorrizadas presentaron una mayor biomasa que las no micorrizadas y una concentración menor de Cu en sus tejidos. Curiosamente, aunque se encontraron algunas diferencias entre el contenido de nutrientes de la parte aérea de las plantas micorrizadas y no micorrizas cultivadas bajo condiciones control, estas diferencias fueron mayores cuando las plantas se cultivaron en suelos contaminados con la concentración más elevada de Cu. El genoma de *Zea mays* contiene 12 genes que codifican potencialmente HMAs, de los cuales cuatro (*ZmHMA3.3, ZmHMA5.1, ZmHMA5.2* y *ZmHMA5.3*) se activan transcripcionalmente por la toxicidad de Cu. Curiosamente, la inducción en la expresión de *ZmHMA3.3* y *ZmHMA5.3* solamente tuvo lugar en la parte aérea y raíz de las plantas micorrizadas. Estos datos sugieren que el desarrollo de la simbiosis bajo condiciones de toxicidad de Cu pude regular los mecanismos de homeostasis de Cu en la planta hospedadora, a través de la inducción específica de ciertas proteínas involucradas en la detoxificación de Cu, potenciando así la tolerancia a Cu de la planta. La predicción de la localización de ZmHMA3.3 y ZmHMA5.3 en el tonoplasto junto con la inducción de su expresión génica en la parte aérea y raíz de las plantas micorrizadas, sugiere que estos transportadores podrían tener un papel dual en la protección frente al Cu de la parte aérea de las plantas micorrizadas, disminuyendo la translocación de Cu a la parte aérea mediante la acumulación de Cu en las vacuolas de las raíces y mediante la compartimentalización vacuolar del exceso de Cu que logra alcanzar la parte aérea. En general, los resultados presentados en el Capítulo IV, realzan la importancia de la simbiosis MA en la tolerancia a metales de la planta hospedadora

En conclusión, los datos presentados en esta tesis doctoral proporcionan un gran avance en el conocimiento de los mecanismos de transporte de Cu en la simbiosis MA, especialmente en la identificación y caracterización de algunos transportadores de Cu del hongo *R. irregularis*. Sin embargo, se necesitan más estudios para entender completamente cómo funciona esta compleja red de proteínas transportadoras en ambos simbiontes, tanto en el hongo como en la planta.

Summary

SUMMARY

The transition metal copper (Cu) is a micronutrient acting as a redox active cofactor of key enzymes involved in a wide array of biochemical processes essential for life, such as respiration, superoxide scavenging, photosynthesis, ethylene perception and iron mobilization. However, when in excess, it becomes toxic due to its ability to displace other metal ions in structural or catalytic protein motifs and through the generation of hydroxyl radicals by Fenton-like reactions which can cause oxidative damage of DNA, lipids and proteins. Due to the dual nature of Cu, organisms have developed sophisticated homeostatic networks to tightly regulate Cu intracellular levels, in which membrane transporters mediating Cu uptake and efflux play a key role. Although Cu is usually present in trace amounts in soils, it can reach toxic levels in certain areas mainly due to anthropogenic activities, such as mining, soil overfertilization and the use of pesticides. Therefore, plants and in general all soil inhabitants have to deal with a wide range of environmental Cu concentrations, from scarcity to excess.

One of the most prominent strategies that plant have evolved to overcome both situations, that is Cu scarcity and toxicity, is the establishment of mutualistic symbioses with arbuscular mycorrhizal (AM) fungi, soil-borne microorganisms belonging to the Glomeromycota. The fungal partner not only colonizes the root cortex but also maintains an extensive and highly branched network of hyphae that extends out the root beyond the depletion zone of nutrients, providing to the plant a new pathway, the mycorrhizal pathway, for the uptake and transport of low mobility nutrients, such as phosphorus, nitrogen, Zn and Cu. Besides improving plant mineral nutrition, AM fungi increase plant tolerance to biotic and abiotic stresses, such as salinity, drought and heavy metal contamination. The ability of arbuscular mycorrhizal fungi to benefit plant growth under Cu deficient and toxic conditions has been reported in many physiological studies. Different mechanisms have been proposed to explain the protective effect of the AM symbiosis in heavy metal stress. However, the mechanisms of Cu transport in the symbiosis are currently unknown.

Within this PhD thesis, with the aim to get further insights into the mechanisms of Cu homeostasis in the AM symbiosis we have used a combination of *in silico*, physiological and molecular approaches, using the symbiosis established between the model AM fungus *Rhizophagus irregularis*, which is easily grown in monoxenic cultures and whose genome sequence is available, and different plant species. *R. irregularis* cultures were established in two experimental systems that allow to obtain exclusively fungal material for molecular analyses, *in vitro* in monoxenic cultures and an *in vivo* whole plant bidimensional experimental system.

As a first step to further understand the mechanisms of Cu homeostasis in the fungal partner, a genome wide analyses was undertaken in order to establish a repertoire of candidate

genes potentially involved in Cu transport. This *in silico* analysis allowed the identification in the genome of *R. irregularis* of seven open reading frames, which potentially encode Cu transporters, belonging to two multigene families. Three candidate genes belong to the CTR family of Cu transporters (*RiCTR1-RiCTR3*) and four to the P_{IB}-ATPase family (*RiCCC2.1-.3* and *RiCRD*). Comparison of these gene families with those of a set of reference fungi revealed an expansion of the *R. irregularis* CCC2 like-ATPases. Analyses of the published transcriptomic profiles of *R. irregularis* showed that *RiCTR2* was highly expressed in mycorrhizal roots, suggesting that Cu is important for fungal colonization. These results are presented in **Chapter I.**

To have some clues into the role of the *R. irregularis* CTRs in the symbiosis, we carried out the first functional characterization of the three putative sequences previously identified in its genome (*RiCTR1, RiCTR2* and *RiCTR3*). These data are presented in **Chapter II**. We have shown that *R. irregularis* expresses two genes encoding Cu transporters of the CTR family, *RiCTR1* and *RiCTR2,* and two alternative spliced variants of a third gene, *RiCTR3*. Functional analyses in yeast revealed that *RiCTR1* encodes a plasma membrane Cu transporter and *RiCTR2* a vacuolar Cu transporter. *RiCTR1* was more highly expressed in the extraradical mycelia (ERM) and *RiCTR2* in the intraradical mycelia (IRM). In the ERM, *RiCTR1* expression was up-regulated by Cu deficiency and down-regulated by Cu toxicity. *RiCTR2* expression increased only in the ERM grown under severe Cu-deficient conditions. These data suggest that RiCTR1 is involved in Cu uptake by the ERM and RiCTR2 in mobilization of vacuolar Cu stores. Cu deficiency decreased mycorrhizal colonization and arbuscule frequency, but increased *RiCTR1* and *RiCTR2* expression in the IRM, which suggests that the IRM has a high Cu demand. The third gene *RiCTR3* produce two alternatively spliced products, *RiCTR3A* and *RiCTR3B*, highly expressed in the ERM. Up-regulation of *RiCTR3A* by Cu toxicity and the capability of its gene product to revert Cu sensitivity of the *Δyap-1* yeast cells suggest that RiCTR3A might function as a Cu receptor necessary to activate downstream signal transduction pathways involved in Cu tolerance.

Once that we showed that Cu uptake in *R. irregularis* ERM is mediated by the plasma membrane Cu transporter RiCTR1, whose gene expression increases under Cu deficiency but decreases under Cu toxicity, we tried to identify the transporter that could be involved in Cu transfer to the plant in the arbuscular interface. Export of metal ions takes place through P_{IB}- type ATPases, also known as heavy metal ATPases (HMA), proteins that couple ATP hydrolysis to the transport of a heavy metal across different cellular membranes. The *Rhizophagus irregularis* genome has four candidate genes putatively encoding P_{IB}-type ATPases. *RiCCC2.1-3* are orthologs of the *Saccharomyces cerevisaie CCC2* Cu-ATPase that transports Cu to Cu containing proteins in the trans-Golgi region and *RiCRD1* is the ortholog of the P_{IB}-ATPase *CaCRD1* of the pathogenic yeast *Candida albicans* that exports Cu excess out of the cell providing Cu resistance. In **Chapter lll**, with the aim of determining whether RiCRD1 plays a role in Cu release to the

apoplast of the symbiotic interface and/or in metal tolerance, the expression patterns of *RiCRD1* were analyzed in the *R. irregularis* IRM and ERM developed in the presence of different Cu levels. Our results strongly suggest that RiCRD could be the transporter responsible for Cu release into the apoplast of the arbuscular interface, as our *in situ* hybridization experiments clearly revealed the presence of *RiCRD* transcripts in the arbuscules developed in the inner cortical cells of mycorrhizal tomato roots. On the other hand, *RiCRD* expression was up-regulated in the ERM by Cu and Cd toxicity. These results are consistent with a role for RiCRD in Cu and Cd detoxification by acting as a metal efflux pump, which agrees with its predicted localization at the plasma membrane. The higher induction of *RiCRD* expression in response to Cu toxicity than of other *R. irregularis* players of Cu tolerance, such as the metallothionein *RiMT* and the ABC transporter *RiABC*, suggests that *RiCRD* is the major determinant of Cu tolerance in *R. irregularis*. Here, we report for the first time a Cu efflux strategy to overcome metal excess in AM fungi. Overall, these data indicate that the Cu exporting ATPase RiCRD could have a dual role in Cu detoxification and symbiotic Cu nutrition, being, therefore, a key player of Cu homeostasis *in R. irregularis*.

Finally, we have analyzed the effect of mycorrhizal inoculation on plant development and metal transport detoxifying mechanisms under Cu toxicity. In **Chapter IV**, we report how inoculation of a Cu sensitive cultivar of *Zea mays* with the AM fungus *R. irregularis* modifies the physiological plant response to Cu toxicity and HMA gene expression. Mycorrhizal plants presented a bigger biomass than non-mycorrhizal plants and less Cu concentration in their tissues. Interestingly, although some differences were found between the shoot nutrient content profiles of mycorrhizal and non-mycorrhizal plants grown under control conditions, these differences were higher in plants grown in soils supplemented with the highest Cu concentration. We found that the *Zea mays* genome harbors 12 genes putatively encoding HMAs, being *ZmHMA3.3, ZmHMA5.1, ZmHMA5.2* y *ZmHMA5.3* up-regulated under Cu toxicity. Interestingly, *ZmHMA3.3* and *ZmHMA5.3* expression was up-regulated only in roots and shoots of mycorrhizal plants. These data suggest that development of the symbiosis under Cu toxic conditions regulates plant Cu homeostasis, through the specific induction of certain proteins involved of Cu detoxification, potentiating in this way plant Cu tolerance. As the *ZmHMA3.3* and *ZmHMA5.3* gene products were predicted to be located at the tonoplast, up-regulation of these genes in roots and shoots of mycorrhizal plants suggests that, these transporters could play a dual role in shoot Cu protection of mycorrhizal plants by preventing root to shoot Cu translocation through its accumulation in the root vacuole and by vacuolar compartmentalization of the excess Cu that reaches the shoot. Overall, data presented in Chapter IV highlight the importance of the AM symbiosis in metal tolerance of their plant hosts.

In conclusion, data presented in this thesis provide a great advance in the knowledge of Cu transport mechanisms in the AM symbiosis, especially in the identification and characterization of some Cu transporters of the AM fungus *R. irregularis*. However, further studies are necessary to completely understand how this complex network of protein transporters function in both the fungal and the plant partners.

Introducción

1. La simbiosis micorrícica arbuscular (MA): generalidades

1.1 Tipos de micorrizas

La palabra micorriza proviene del griego "*mycos*" hongo y "*riza*" raíz, tal y como su etimología sugiere, las micorrizas son una asociación simbiótica mutualista que establecen ciertos hongos del suelo con las raíces de las plantas. Se trata de una asociación altamente interdependiente en la que, básicamente, la planta huésped obtiene del hongo agua y nutrientes mientras que a cambio el hongo recibe de la planta compuestos carbonados necesarios para completar su ciclo vital (Lanfranco et al., 2016). Además, la planta hospedadora mejora su resiliencia frente a estreses bióticos y abióticos y no solo como resultado de la mejora en su estatus nutricional, como se detallará más adelante (Jung et al., 2012; Auge et al., 2015; Ferrol et al., 2016). Los beneficios de esta simbiosis son tales que la mayoría de las plantas se encuentran micorrizadas de forma natural en los ecosistemas (Brundrett and Tedersoo, 2018).

Se reconocen diferentes tipos de micorrizas en las que están implicados distintos grupos de hongos y plantas huésped, con patrones morfológicos claramente distinguibles. Se diferencian tres tipos fundamentalmente: las ectomicorrizas, las endomicorrizas y las ectendomicorrizas, siendo estas últimas un tipo intermedio entre las anteriores (**Figura 1**). Las ectomicorrizas y las micorrizas arbusculares, son los dos tipos más representativos y estudiados, por su importancia forestal y agrícola, respectivamente.

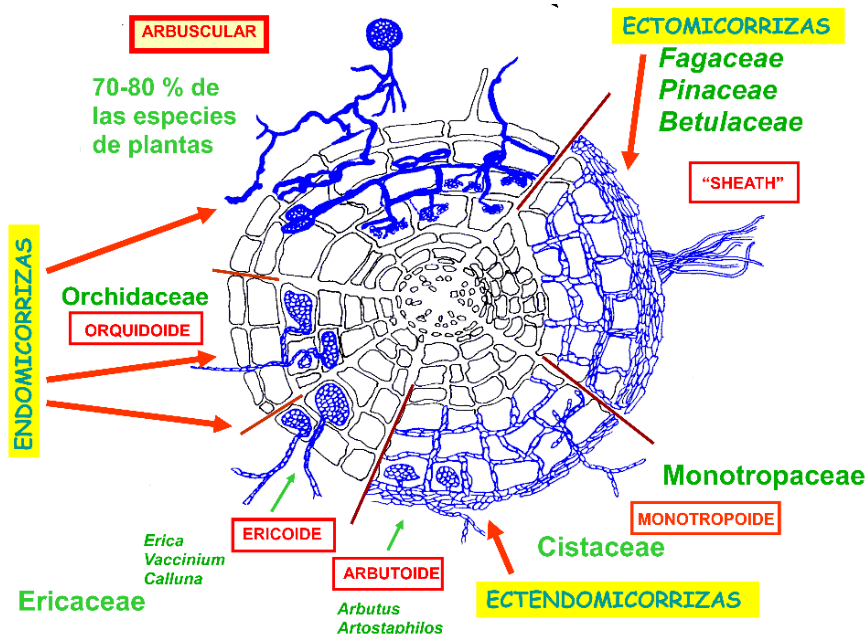


Figura 1. Principales tipos de micorrizas. Los tres tipos principales de micorrizas están resaltados en amarillo, los subtipos en rojo y las plantas hospedadoras en verde. Esquema cedido por José Miguel Barea.

Aunque solo el 2% de las plantas vasculares forman ectomicorrizas, en su mayoría gimnospermas, entre éstas se encuentran árboles de gran importancia económica y ecológica, pertenecientes a familias como *Pinaceae*, *Fagaceae*, *Betulaceae*, *Nothofagaceae*, *Dipterocarpaceae* y *Caesalpinaceae* (Tedersoo *et al.,* 2010). Los hongos responsables de esta asociación son mayoritariamente Basidiomicetes y algunos Ascomicetes y Zigomicetes, entre los que se encuentran hongos comestibles. Se caracterizan por la formación de un entramado compacto de hifas denominado "manto", que rodea la superficie de las raíces. Por otro lado, las hifas penetran en las raíces, pero no en el interior de las células, ocupando los espacios intercelulares entre las células epidérmicas y corticales formando la denominada "red de Hartig" donde se produce el intercambio de nutrientes entre ambos simbiontes (Brundrett and Tedersoo, 2018).

Las endomicorrizas se caracterizan por no formar manto fúngico y porque las hifas penetran intracelularmente en las células corticales de la raíz. El 10% de las plantas vasculares forman simbiosis endomicorrícicas de tipo orquidoide y el 1.5% de tipo ericoide, estando su formación restringida a determinadas familias vegetales, *Orchidaceae* y *Ericaceae,* respectivamente. Las micorrizas arbusculares, también endomicorrícicas, constituyen el tipo de simbiosis más ampliamente distribuida por todo el reino vegetal (Brundrett and Tedersoo, 2018).

La simbiosis micorrícica arbuscular (MA), en la que se centra el presente estudio, la establecen hongos del suelo pertenecientes al filo Glomeromycota y las raíces de la mayoría de las plantas, incluyendo el 71% de las plantas vasculares, muchas de ellas con importancia agrícola. Desgraciadamente *Arabidopsis*, ampliamente utilizada como planta modelo en estudios genéticos, no se micorriza. Esta simbiosis también está presente en plantas criptógamas más primitivas, como el 67% de helechos y el 25% de los briófitos (Brundrett and Tedersoo, 2018). Se caracteriza por la formación de unas estructuras intracelulares especializadas altamente ramificadas en forma de árbol, denominadas arbúsculos, dentro de las células corticales de la raíz que no llegan a romper la membrana plasmática, la cual se invagina entorno a ellos. Esta membrana de origen vegetal, denominada membrana periarbuscular, junto con la membrana plasmática del hongo, consta de una composición única de proteínas transportadoras para permitir el intercambio bidireccional de nutrientes entre los simbiontes, que se produce en la interfase apoplástica que queda entre ellas o espacio periarbuscular. Por ello, los arbúsculos se consideran estructuras clave en la simbiosis MA (Luginbuehl and Oldroyd, 2017; MacLean et al., 2017) (**Figura 2**).

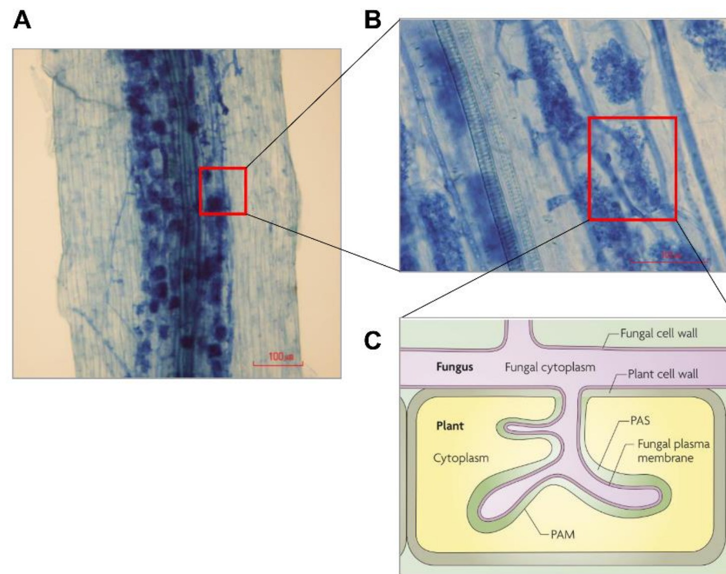


Figura 2. Micorrizas arbusculares. (A) Raíz micorrizada de achicoria (*Cichorium intybus* L.) con el hongo MA *Rhizophagus irregularis* DAOM 197198 (B) Detalle de los arbúsculos en esa misma raíz. (C) Esquema basado en Parniske (2008) de una célula cortical colonizada por un arbúsculo, en el que se indican los componentes de la interfase simbiótica. PAM: membrana periarbuscular (del inglés, periarbuscular membrane), PAS: espacio periarbuscular (del inglés, periarbuscular space). Barra de escala 100 µm.

Finalmente, cabe señalar que se reconocen dos tipos de micorrizas arbusculares: *Paris* y *Arum*. El tipo *Paris* se caracteriza por presentar hifas intracelulares y "rulos arbusculares". En cambio, en el tipo *Arum* las hifas crecen intercelularmente y penetran en las células corticales formando los típicos arbúsculos. Ambos tipos morfológicos dependen de la combinación entre la especie fúngica y vegetal (Dickson 2004). Aunque el tipo *Paris* es el más frecuente en la naturaleza, la mayoría de los genes simbióticos vegetales han sido caracterizados en micorrizas tipo *Arum*, el más extendido entre las plantas cultivables (Dickson, 2004; Cosme et al., 2018). Incluso es posible la coexistencia de los dos tipos como se ha descrito para el tomate y el pepino (Kubota et al., 2005).

1.2 Evolución y filogenia de los hongos MA

El origen de los hongos MA se cree que fue en el Ordovicídico, hace más de 400 millones de años y fueron clave en el proceso de colonización de los ecosistemas terrestres por las plantas, tal y como sugirieron Pirozynski y Malloch ya en 1975 (Pirozynski and Malloch, 1975). El registro fósil más antiguo data de hace 460 millones de años, consistente en esporas e hifas muy similares a las de los hongos MA actuales aunque no se encontraron en asociación con restos vegetales (Redecker et al., 2000). Sin embargo, los primeros fósiles bien preservados de la simbiosis MA son rizomas micorrícicos de hace 407 millones de años encontrados en el yacimiento del Devónico de Rhynie Chert, Escocia (Taylor et al., 1995; Krings et al., 2007; Brundrett and Tedersoo, 2018). Por otro lado, algunos genes simbióticos preceden a la aparición

de las plantas terrestres (Delaux, 2017; Martin et al., 2017). Debido a los escasos registros fósiles durante los primeros 60 años de vida en la tierra, se ha cuestionado si los hongos micorrícicos se originaron con las primeras plantas terrestres en el Ordovicídico o con el rápido aumento en la complejidad vegetal que tuvo lugar en el Silúrico (Brundrett and Tedersoo, 2018).

Se distinguen tres episodios clave en la evolución de las micorrizas, el primero coincide con el anteriormente mencionado, es decir, con la aparición de las micorrizas arbusculares en las primeras plantas terrestres. El segundo y el tercero están asociados a cambios climáticos, así como a un aumento en la complejidad de los hábitats y los suelos, que determinó una ventaja competitiva a ciertos tipos de raíces más especializados que combinan incluso micorrizas de diferentes tipos, micorrizas arbusculares y ectomicorrizas, en la misma raíz. Concretamente, el segundo episodio coincide con el Cretácico, con la aparición de las ectomicorrizas, las micorrizas orquidioides y ericoides, así como linajes de plantas no micorrícicas con otras estrategias nutricionales, mientras que el tercero está teniendo lugar en la actualidad asociada a la rápida diversificación vegetal en los puntos calientes de biodiversidad (Brundrett and Tedersoo, 2018). Las transiciones entre estrategias de nutrición son más frecuentes de lo que se pensaba, siendo las ectomicorrizas el tipo de micorriza más frecuentemente ganado o perdido durante la evolución. Actualmente se estima que solo el 8% de las plantas vasculares no están micorrizadas, muchas de ellas especialistas de hábitat que crecen en ambientes donde la productividad está limitada por las condiciones del suelo o del clima, como las epífitas, o especialistas nutricionales como como plantas carnívoras o parásitas (Brundrett and Tedersoo, 2018). Una información más extensa sobre la coevolución de los hongos micorrícicos con las plantas se puede encontrar en varias revisiones (Bonfante and Genre, 2008; Martin et al., 2017; Brundrett and Tedersoo, 2018).

Los intentos de clasificar los hongos MA se remontan a finales del siglo XIX - principios del XX, cuando en base a criterios morfológicos se incluyeron en la familia *Endogonazeae*, en el filo polifilético Zigomycota. La incorporación de técnicas moleculares a los estudios filogenéticos de los hongos MA permitieron a Schüβler y colaboradores (2001), mediante el análisis de la subunidad pequeña del ARNr, separarlos en un nuevo filo Glomeromycota, de origen monofilético. Sin embargo, en el 2016 Spatafora y colaboradores determinaron que los Zigomycetes no eran un grupo polifilético sino parafilético y lo dividieron en dos filos Zoopagomycota y Mucoromycota, incluyendo a los hongos MA dentro de este último, pasando así a la categoría de subfilo (Glomeromycotina). No hay consenso en esta clasificación y recientemente los hongos MA se han separado de nuevo en el filo Glomeromycota (Wijayawardene et al., 2018) (**Figura 3**).

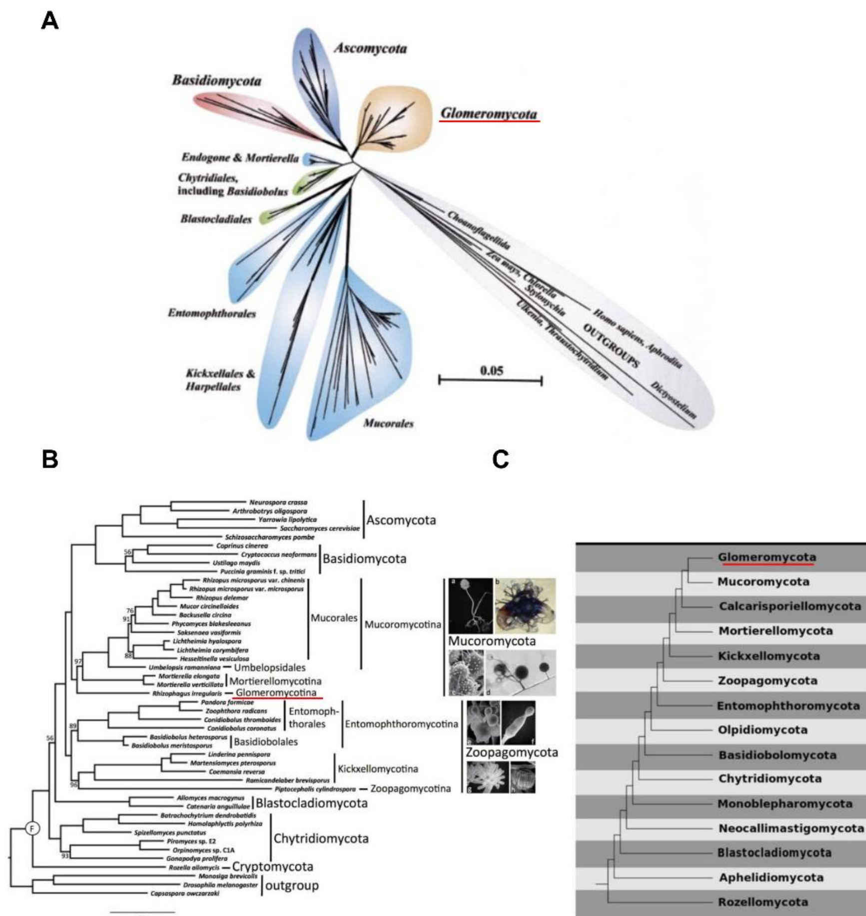


Figura 3. Filogenias propuestas para los hongos MA en el reino Fungi (A) Filogenia propuesta por por Schüβler y colaboradores (2001), el cual identifica a los hongos MA como un filo Glomeromycota de origen monofiletico (B) Filogenia propuesta por (Spatafora et al., 2016) en la que los hongos MA bajan a categoría de subfilo Glomeromycotina, dentro del filo Mucoromycota (C) Filogenia actual, los hongos MA se reconsideran como filo (Wijayawardene et al., 2018). La posición de los hongos MA está subrayada en rojo en todas las filogenias.

La clasificación dentro del filo Glomeromycota también ha variado a lo largo del tiempo con la incorporación de las técnicas moleculares en la identificación de las especies, tradicionalmente basada exclusivamente en caracteres morfológicos asociados a las esporas, tales como su tamaño, coloración, forma, etc. En 2013 Redecker y colaboradores presentaron una clasificación consenso dentro del filo Glomeromycota (**Figura 4A**). Actualmente este filo está representado por 1 clase, 4 órdenes, 12 familias, 34 géneros y 312 especies (lista actualizada en Mayo de 2018 de la página web http://www.amf-phylogeny.com/amphylo_taxonomy.html) (**Figura 4B**), aunque puede variar en función de la clasificación consultada.

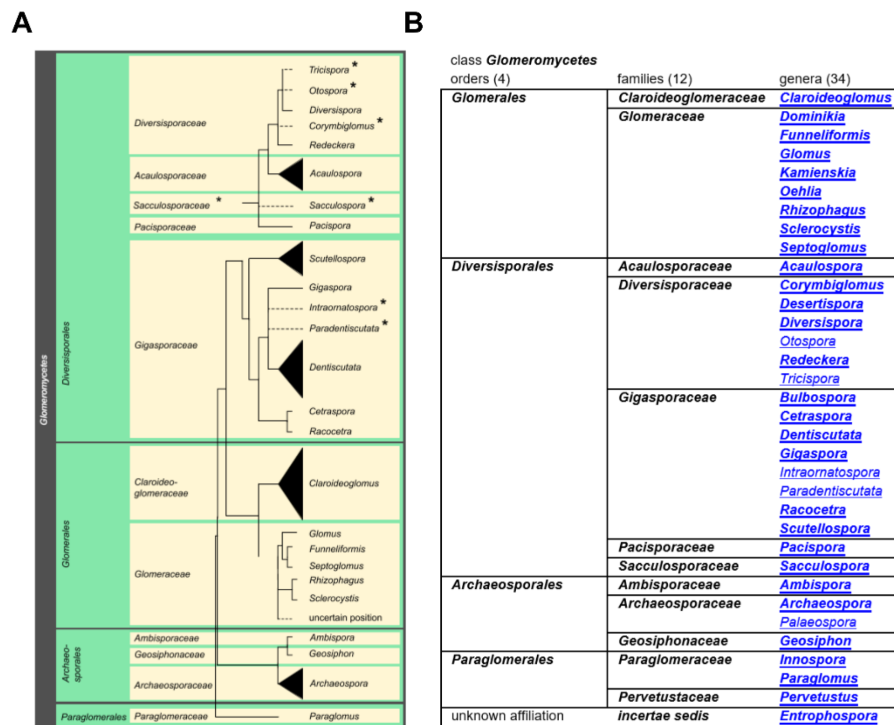


Figura 4. Clasificación de los hongos MA (A) Clasificación consenso para el filo Glomeromycota propuesta por (Redecker et al., 2013) (B) Clasificación actualizada obtenida de la página web http://www.amf-phylogeny.com/amphylo_taxonomy.html (última modificación, Mayo 2018).

1.3 Características generales de los hongos MA

La característica más notable y que dificulta enormemente su estudio, es su biotrofía obligada, es decir, solo pueden crecer en asociación con una planta hospedadora. Hasta la fecha, no se han conseguido cultivar sin la planta hospedadora, por lo que es difícil la obtención de material fúngico. Sin embargo, es posible cultivarlos *in vitro* en cultivo monoxénico con raíces transformadas con *Agrobacterium rhizogenes*, permitiendo la obtención de material fúngico libre de material vegetal y de otros microorganismos, aunque este tipo de cultivo aún no está adaptado para todas las especies de hongos MA. Esto hace que la mayoría de estudios moleculares estén centrados en *Rhizophagus irregularis*, uno de los hongos que mejor crece en cultivo monoxénico (St-Arnaud et al., 1996; Sanders and Croll, 2010). Además de esta característica, los hongos MA tienen otras peculiaridades que les hacen ser organismos altamente complejos en su estudio.

Poseen hifas cenocíticas con un citoplasma común y esporas multinucleadas. El número de núcleos por espora es variable, aproximadamente entre 700 a 35 000 núcleos, en función de la especie de hongo MA (Hosny et al., 1998). Se reproducen asexualmente, sin embargo, los núcleos pueden viajar por las hifas (**Figura 5**) y ser transferidos de un micelio a otro genéticamente diferente mediante procesos de anastomosis, con una frecuencia inversamente proporcional a la distancia genética, por lo que limitaría el intercambio de núcleos a especies estrechamente relacionadas (Sanders, 1999; Croll et al., 2009; Lanfranco and Young, 2012). Este proceso de

anastomosis podría justificar los polimorfismos que se han encontrado a nivel de secuencia de un mismo gen en un mismo aislado o entre aislados diferentes en ausencia de recombinación génica (Vandenkoornhuyse et al., 2001; Croll and Sanders, 2009; den Bakker et al., 2010; Lanfranco and Young, 2012). Sin embargo, el descubrimiento de la presencia de genes implicados en la meiosis en el genoma de *R. irregularis* (Halary et al., 2011; Tisserant et al., 2012; Tisserant et al., 2013) y, más recientemente, de genes tipo MAT (mating-type) relacionados con el apareamiento fúngico en Dikarya (Ropars et al., 2016; Corradi and Brachmann, 2017) alejan la visión de que los hongos MA sean simples organismos clonales sin capacidad de reproducirse sexualmente.

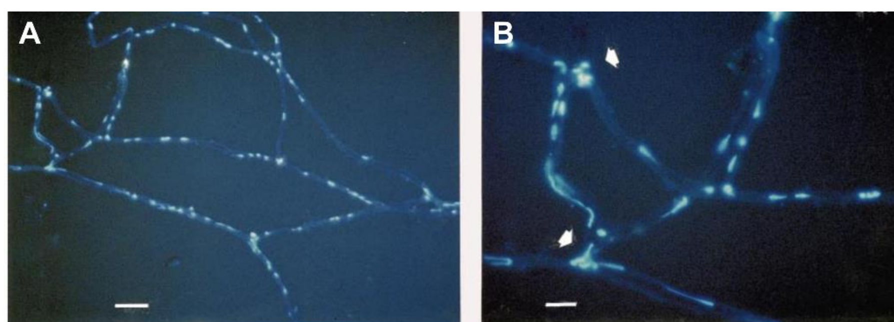


Figura 5. Anastomosis en el micelio extraradical del hongo MA *Funeliformis mosseae.* (A) Visión general del proceso de anastomosis mediante microscopía de epifluorescencia, los núcleos están teñidos con DAPI (B) Visión más detallada, las flechas señalan núcleos que se encuentran en las zonas de fusión entre hifas. Barra de escala A: 25µm; B: 10µm (Giovannetti et al., 2001).

Su biotrofía obligada y la presencia de esporas y micelio con múltiples núcleos, limitan la aplicación de técnicas moleculares normalmente aplicadas para el estudio de la funcionalidad génica. No hay protocolos de transformación genética estable para hongos MA, de manera que es imposible la obtención de mutantes "knockout" deficientes en un gen de interés, y tradicionalmente los estudios de caracterización funcional se han llevado a cabo en sistemas heterólogos en levadura (Sanders, 1999). Ya se han utilizado estrategias de silenciamiento génico "host induced gene silencing" (HIGS) y "virus induced gene silencing" (VIGS) para la caracterización de genes del hongo, aunque no siempre se han aplicado con éxito, sobre todo para genes con niveles bajos de expresión *in planta* (Lanfranco and Young, 2012; Groten et al., 2015; Tsuzuki et al., 2016; Xie et al., 2016; Sun et al., 2018; Voß et al., 2018).

Finalmente, los hongos MA pueden albergar bacterias endosimbiontes cuyo origen y función todavía no están claros (Bianciotto et al., 2000; Bianciotto et al., 2003; Naumann et al., 2010; Desiro et al., 2014). Lumini y colaboradores (2007) demostraron que la curación de β-proteobacterias endosimbiontes de *Gigaspora margarita* afectaba a la morfología de las esporas y comprometía el crecimiento presimbiótico del hongo. Esto sugiere que las bacterias endosimbiontes también podrían jugar un papel relevante en la simbiosis MA y, sin duda, añaden otro nivel de complejidad a su estudio.

1.4 El ciclo de vida de los hongos MA

El desarrollo de la simbiosis MA puede separarse en diferentes etapas (**Figura 6**). Sin embargo, se trata de un proceso altamente asincrónico, en el sentido de que nos podemos encontrar todas las estructuras fúngicas (hifopodio, hifas intracelulares, arbúsculos etc.) presentes simultáneamente en una misma raíz, lo que dificulta su estudio (Pimprikar and Gutjahr, 2018). Este proceso de colonización de la raíz por los hongos MA es dependiente del estado de desarrollo y condición fisiológica de la planta hospedadora. Los mecanismos de señalización y de regulación requeridos para el desarrollo de la simbiosis MA han sido revisados recientemente (Luginbuehl and Oldroyd, 2017; MacLean et al., 2017; Pimprikar and Gutjahr, 2018) y se resumen a continuación.

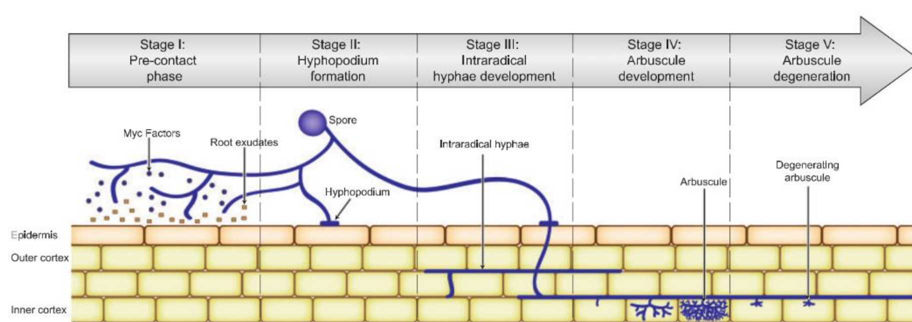


Figura 6. Etapas clave en el desarrollo de la simbiosis MA. En la fase presimbiotica (etapa I) aún no existe contacto físico entre los simbiontes y se caracteriza por el establecimiento de un diálogo molecular mediante moléculas difusibles que permite el reconocimiento entre los simbiontes. La fase simbiótica está representada por las etapas I-V en la figura. Imagen de (Pimprikar and Gutjahr, 2018).

Antes del contacto físico, se requiere el reconocimiento mediante moléculas difusibles por parte de ambos simbiontes. Esta etapa normalmente es conocida como etapa de pre-contacto o etapa pre-simbiótica. Las plantas, especialmente bajo condiciones limitantes de fosfato inorgánico, liberan estrigolactonas que estimulan la germinación de las esporas de los hongos MA, su actividad metabólica, así como la elongación y ramificación de las hifas de los hongos MA. Las estrigolactonas igualmente son utilizadas como señal para la germinación de plantas parásitas, como las pertenecientes a los géneros *Striga* y *Orobanche* (López-Ráez et al., 2011; Waters et al., 2017). Las esporas de los hongos MA pueden experimentar múltiples y sucesivas rondas de germinación y retracción de sus hifas antes de encontrar una raíz susceptible de ser colonizada (Azcón-Aguilar et al., 1999).

Por su parte, los hongos MA liberan oligómeros de quitina denominados factores Myc (“Myc factors”) que incluyen lipo-quito-oligosacáridos (Myc-LCOs) similares a los factores Nod liberados por rizobacterias fijadoras de nitrógeno y otros de cadena más corta tetra o penta-quito-oligiosacáridos (Myc-COs). Estos factores Myc son reconocidos por receptores de la planta y desencadenan picos de Ca^{2+} nucleares similares a los que se producen en la señalización

simbiótica *Rizobium*-leguminosa, por lo que se cree que parte de la ruta de señalización, conocida como “common symbiosis pathway” (CSP), podría ser compartida por ambas simbiosis, aunque muchos de los componentes de esta ruta aún están por descubrir (Oldroyd, 2013; Genre and Russo, 2016). El significado biológico de los factores Myc aún no está claro, aunque existen indicios de que diferentes plantas podrían diferir en la capacidad de percepción de estos factores (Sun et al., 2015a) y que la composición del coctel de factores Myc podría diferir de unos hongos MA a otros o incluso durante su ciclo de vida (Lanfranco et al., 2018). También se han descrito compuestos orgánicos volátiles fúngicos (VOCs) que pueden reprogramar la arquitectura de la raíz e influir en el sistema de defensa de la planta hospedadora (Sun et al., 2015b; Werner et al., 2016). El sistema de comunicación entre los simbiontes es complejo y aún queda mucho por descubrir (Lanfranco et al., 2018).

La fase simbiótica comienza cuando una hifa logra tocar la epidermis de la raíz y forma una estructura de adhesión denominada hifopodio o apresorio. La planta facilita la entrada del hongo mediante la formación de una estructura transcelular tubular denominada aparato de pre-penetración (PPA) que guía el avance intracelular de las hifas hasta llegar a las células del cortex donde forman los arbúsculos, estructuras intracelulares clave en el intercambio de nutrientes entre ambos simbiontes (Genre et al., 2008). Estos quedan rodeados por la membrana periarbuscular, de origen vegetal, que junto con la membrana del arbúsculo forman una amplia interfase para el intercambio bidireccional de nutrientes (MacLean et al., 2017). Los arbúsculos son estructuras dinámicas y con el tiempo se degeneran y se forman nuevos arbúsculos.

Paralelamente a este desarrollo en el interior de la raíz el hongo desarrolla una red de micelio extrarradical. Inicialmente, desarrolla unas hifas relativamente gruesas, llamadas hifas exploradoras, que son las responsables del avance del micelio y de la expansión de la colonia fúngica. Estas hifas producen ramificaciones periódicas, las hifas secundarias, que se ramifican sucesivamente (Bago et al., 1998b). Sobre las hifas se forman los “BAS” (del inglés, branched absorbing structures) que son estructuras ramificadas cuya hipotética función es la absorción de los nutrientes del suelo (Bago et al., 1998a; Bago, 2000). Los BAS son estructuras transitorias y al cabo de unos días retraen su citoplasma, a menos que desarrollen esporas, cerrándose de este modo el ciclo de vida del hongo.

1.5 Beneficios de la simbiosis MA

Los beneficios de la simbiosis MA se sustentan en el intercambio bidireccional de nutrientes entre la planta el hongo MA. Ambos simbiontes se ven altamente recompensados, como es característico de las asociaciones mutualistas.

1.5.1 Beneficios obtenidos por el hongo

El hongo depende del suministro de compuestos carbonados por parte de la planta hospedadora, tradicionalmente se pensaba que este aporte de carbono se producía en forma de azúcares, pero estudios recientes apuntan a que también se produce en forma de ácidos grasos, ya que los hongos MA son auxótrofos en ácidos grasos y dependen completamente de la planta hospedadora para su suministro. Este descubrimiento surgió con la publicación del genoma y transcriptoma de las primeras especies de hongos MA (Tisserant et al., 2012; Tisserant et al., 2013; Lin et al., 2014; Kamel et al., 2016), que puso de manifiesto la ausencia de genes que codifican el complejo FAS (del inglés, fatty acid synthase) que está conservado en eucariotas y es esencial para la síntesis de ácidos grasos *de novo* (Wewer et al., 2014; Tang et al., 2016). Esta idea fue apoyada con otras evidencias experimentales como el hecho de que en plantas mutantes de leguminosas que carecían de genes los *DIS, FatM, RAM2,* implicados en la biosíntesis de lípidos e inducibles por la micorrización, se formaban arbúsculos poco desarrollados y la colonización se reducía (Wang et al., 2012; Pimprikar et al., 2016; Bravo et al., 2017; Jiang et al., 2017; Keymer and Gutjahr, 2018). Esta alta dependencia por los lípidos suministrados por la planta explicaría la alta cantidad de lípidos que acumulan en sus esporas y que ayudarían a sustentarse en su etapa presimbiótica, hasta que con el desarrollo de los primeros arbúsculos pudiesen obtener lípidos de la planta (Lanfranco et al., 2018). Por otro lado, los hongos MA parecen tener cierta capacidad de tomar pentosas y hexosas del medio mediante transporte activo como demostraron Helber y colaboradores (2011) con sustratos marcados con C^{14}. Actualmente se piensa que esta total dependencia en el aporte de lípidos por parte de la planta hospedadora probablemente sea la principal razón de su biotrofia obligada (Lanfranco et al., 2018). Sin embargo, todavía no se han identificado los transportadores implicados en dicha transferencia.

Por otro lado, la transferencia de C en forma de azúcares ha sido estudiada durante muchos años. A nivel de la planta, se ha observado que algunos genes que codifican transportadores de sacarosa (SUTs), de monosacáridos (MSTs) y, más recientemente, miembros de la familia SWEET (Manck-Gotzenberger and Requena, 2016) se inducen en raíces micorrizadas. Sin embargo, aún no hay evidencias experimentales suficientes que hayan permitido identificar las proteínas implicadas en dicha transferencia (Doidy et al., 2012; Garcia et al., 2016; Lanfranco et al., 2018). A nivel del hongo, el transportador de monosacáridos de alta afinidad *RiMST2* de *R. irregularis*, que se expresa tanto en las hifas intraradicales como en los

arbúsculos, y cuyo silenciamiento da lugar a una reducción en la colonización micorrícica y a la aparición de arbúsculos truncados, es el transportador fúngico responsable de la captación de monosacáridos, especialmente glucosa, presente en la interfase simbiótica (Helber et al., 2011).

La planta hospedadora destina hasta el 20% del C que fija por la fotosíntesis para el simbionte fúngico (Roth and Paszkowski, 2017), pero esta gran inversión de C se ve altamente compensada por los beneficios que la simbiosis proporciona a la planta, como se detallará a continuación.

1.5.2 Beneficios obtenidos por la planta hospedadora

El principal beneficio que obtiene la planta es nutricional. El hongo mejora notablemente la adquisición de agua y de macronutrientes de baja movilidad en el suelo, especialmente del fósforo y algunos micronutrientes como cobre y zinc (Javot et al., 2007b; Smith et al., 2011; Ferrol et al., 2018). En gran medida se debe a que el micelio externo incrementa enormemente el volumen de suelo que puede ser explorado por la raíz, más allá de la zona de depleción de nutrientes. Por lo tanto, las plantas micorrizadas disponen de dos vías para la captación de nutrientes, la vía directa a través de las células epidérmicas de la raíz y la vía micorrícica a través de sus simbiontes fúngicos (**Figura 7**). En el caso del fósforo, se ha estimado que entre el 20-100% podría ingresar en la planta por vía micorrícica (Smith et al., 2011). La mayoría de estudios se han centrado en la captación de macronutrientes, como el fósforo y nitrógeno, en los que se han caracterizado gran parte de los transportadores implicados, tal y como se detallará en el siguiente aparatado.

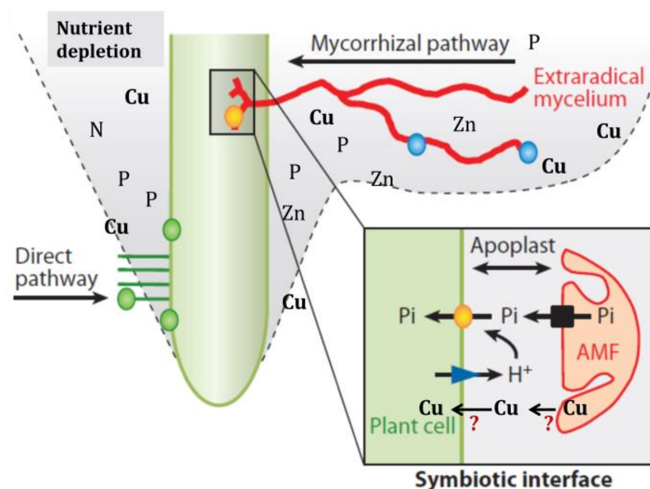


Figura 7. Representación esquemática de las vías de adquisición de nutrientes en plantas micorrizadas. Imagen modificada de (Smith and Smith, 2011)

Sin embargo, a pesar de las numerosas evidencias de que las micorrizas mejoran también la captación de micronutrientes (Li et al., 1991; Liu et al., 2000; Pellegrino et al., 2015) se sabe muy poco acerca de cómo se produce este transporte, sobre todo por parte del simbionte fúngico debido a la alta complejidad de estudio que suponen los hongos MA.

Por otro lado, además del beneficio nutricional que obtiene la planta hospedadora, se ha demostrado que las plantas micorrizadas incrementan su tolerancia frente a estreses bióticos y abióticos en comparación con las no micorrizadas y que estos efectos no pueden ser explicados solo como consecuencia del mejor estatus nutricional de la planta hospedadora. Así, se ha demostrado el papel de bioprotección de las micorrizas frente al ataque de numerosos microorganismos patógenos e insectos tanto atacantes de la parte radicular como aérea, con un efecto variable en función del estilo de vida de su atacante (Jung et al., 2012; Pozo et al., 2013). También se ha demostrado su beneficio frente a estrés por salinidad y sequía (Ruiz-Lozano, 2003; Aroca et al., 2007; Quiroga et al., 2017; Balestrini et al., 2018), así como al estrés por metales pesados (Schützendübel and Polle, 2002; Ferrol et al., 2016), siendo este último objeto de nuestro estudio y que se detallará en el siguiente apartado.

A nivel ecosistémico la simbiosis MA también tiene gran importancia. Además de su evidente impacto en el ciclo biogeoquímico de los nutrientes, también mejoran la calidad del suelo (Rillig et al., 2014), modulan la composición de la microbiota de la rizosfera (Barea et al., 2005; Artursson et al., 2006) y modifican la estructura y composición de las comunidades vegetales (Klironomos et al., 2000; van der Heijden et al., 2008; Wagg et al., 2011; Cipollini et al., 2012). Por otro lado, la abundancia de hospedadores susceptibles de ser micorrizados y la posibilidad de anastomosis entre las hifas del micelio externo, abre la posibilidad a que distintas especies vegetales puedan estar conectadas a nivel de sus raíces e intercambiar recursos e información (Giovannetti et al., 2001; Bago et al., 2002; de la Providencia et al., 2005). Por todo esto, las micorrizas se consideran elementos clave que afectan al funcionamiento y dinámica de los ecosistemas.

1.6 Mecanismos de transporte de nutrientes en la simbiosis MA

La importancia de la simbiosis MA en la nutrición fosforada y nitrogenada de la planta ha sido ampliamente estudiada, y gran parte de los transportadores implicados en la transferencia de fósforo y nitrógeno por parte de ambos simbiontes han sido caracterizados. Revisiones actuales detallan los últimos avances en el transporte de estos macronutrientes (Ferrol et al., 2018; Lanfranco et al., 2018; Wipf et al., 2019) y se resumen a continuación.

El fósforo es uno de los principales macronutrientes que limita el crecimiento vegetal debido a su baja movilidad en el suelo, en el que normalmente se encuentra en forma de iones dihidrógeno fosfato $H_2PO_4^-$. Esto hace que enseguida se genere una zona de depleción o agotamiento de fósforo alrededor de las raíces. En respuesta, las plantas activan una serie de mecanismos para optimizar su adquisición, entre ellos, favorecen su micorrización mediante la liberación de estrigolactonas, como se ha comentado anteriormente (López-Ráez et al., 2011). Algunas especies de plantas pueden llegar a adquirir casi la totalidad del fósforo por la vía micorrícica (Smith et al., 2003).

La captación de fósforo a través del micelio extrarradical del hongo ocurre a través de cotransportadores fúngicos $Pi:H^+$ (PT) homólogos al transportador de alta afinidad PHO84 de la levadura *Saccharomyces cerevisiae* (Lanfranco et al., 2018). Se han caracterizado en varias especies de hongos MA, como en *Diversispora epigaea* (anteriormente, *Glomus versiforme*) (DePT; Harrison and Buuren, 1995), *F. mosseae* (anteriormente, *Glomus mosseae*) (FmPT; Benedetto et al., 2005), *R. irregularis* (RiPT; Maldonado-Mendoza et al., 2001) y más recientemente en *Gigaspora margarita* (GigmPT; Xie et al., 2016) al que se le ha atribuido una función de transceptor, es decir, una función dual como transportador y receptor de Pi (Kriel et al., 2011). Una vez que el Pi es absorbido, es rápidamente almacenado en forma de cadenas de polifosfato (PoliP) en las vacuolas y translocado al micelio intrarradical, concretamente a los arbúsculos (Ezawa et al., 2004; Hijikata et al., 2010). El PoliP es hidrolizado de nuevo a Pi a través de la acción de fosfatasas y exportado al espacio periarbuscular a través de mecanismos aún desconocidos. La planta toma el Pi a través de transportadores que se encuentran en la membrana periarbuscular, inducibles por micorrización, y que han sido identificados y caracterizados en numerosas especies vegetales como MtPT4 en *Medicago truncatula,* OsPT11 en arroz y ZmPth1;6 en maíz, entre otras (Harrison et al., 2002; Javot et al., 2007a; Yang et al., 2012). La importancia de la vía micorrícica en la toma de Pi queda reflejada en la represión de la expresión de transportadores presentes en las células epidérmicas de la raíz implicados en la vía directa de captación de Pi desencadenada por la micorrización de las plantas (Liu et al., 1998).

El transporte de micronutrientes se abordará en la siguiente sección.

2. Estrés por cobre

2.1 La bioquímica del cobre (esencialidad vs. toxicidad)

Algunos metales pesados, entre ellos el cobre (Cu), son micronutrientes esenciales, pero en exceso son altamente tóxicos. El Cu, debido a sus propiedades redox, es usado como cofactor por muchas cuproproteinas que intervienen en procesos esenciales para la vida, como en la respiración (citocromo c oxidasa), la fotosíntesis (plastocianina), la eliminación de especies reactivas de oxígeno ROS (superóxido dismutasa), el trasporte de Fe (multicobre oxidasas), la percepción del etileno (receptor de etileno ETR1), etc., (Linder, 1991; Festa and Thiele, 2011). Sin embargo, en exceso el Cu es altamente tóxico debido a su capacidad de desplazar otros cofactores metálicos presentes en proteínas (Macomber and Imlay, 2009) y a través de la generación de radicales hidroxilos por reacciones tipo Fenton, que pueden causar daño oxidativo en el ADN, lípidos y proteínas (Halliwell and Gutteridge, 1984). Esta naturaleza dual del Cu, hace que todos los organismos dispongan de redes homeostáticas altamente reguladas que les permitan mantener los niveles de Cu intracelulares equilibrados y en las que las proteínas transportadoras de Cu, pertenecientes a diferentes familias multigénicas y que median la adquisición y el eflujo de Cu, juegan papeles clave, como se detallará en los siguientes apartados. Aunque el Cu suele ser un elemento traza en los suelos, también puede encontrarse en exceso en determinadas áreas de forma natural o como consecuencia de actividades antropogénicas como la minería, la sobrefertilización y el uso de pesticidas, que han provocado la contaminación de los suelos con este metal. Así las plantas, y en general todos los organismos y microorganismos que habitan el suelo tienen que hacer frente a concentraciones de Cu variables, tanto de deficiencia como de toxicidad; así como a una demanda de este micronutriente que también puede ser variable en función de los diferentes tejidos o etapa de desarrollo. La simbiosis MA es beneficiosa para la planta hospedadora en ambas situaciones, tanto en condiciones limitantes como de exceso de metales (Ferrol et al., 2016).

2.2 Mecanismos de homeostasis de Cu

El transporte de Cu al interior de la célula constituye el primer paso de los mecanismos de homeostasis del metal a nivel celular y, por tanto, su control resulta crítico para regular los niveles intracelulares de dicho elemento. Las células eucariotas disponen de una amplia variedad de transportadores metálicos con diferente especificidad de sustrato y propiedades cinéticas. La adquisición de Cu del medio en eucariotas está mediada por transportadores pertenecientes a la familia CTR (del inglés, copper transporter) (Puig, 2014). Una vez en el interior celular, debido a la limitada solubilidad y a la gran reactividad del Cu, éste se une a pequeñas proteínas citosólicas denominadas chaperonas que actúan como carabinas moleculares y lo distribuyen en los diferentes emplazamientos hacia sus dianas específicas, para finalmente ser incorporado en

cuproproteínas biológicamente activas que requieren el Cu como cofactor (Huffman y O'Halloran, 2001). Se han descrito diferentes chaperonas tales como las proteínas Ccs (del inglés, copper chaperone for superoxide dismutase), Cox17 (citocromo oxidasa 17) y Atx1 (proteína antioxidante 1). Para una completa distribución subcelular del metal, es necesaria la presencia de transportadores en las membranas intracelulares a los que las chaperonas proporcionarían el Cu para bombearlo finalmente a los compartimentos destino, tales como P_{-1B} ATPasas de metales pesados (HMA, del inglés heavy metal ATPases) implicadas en la distribución de metales desde el citosol a través de las membranas intracelulares mediante la hidrólisis de ATP, como las del tipo CCC2 (Yuan et al., 1997). Otros miembros de esta misma familia pueden estar presentes en la membrana plasmática y mediar la expulsión del exceso de metales hacia el exterior (Williams and Mills, 2005). Adicionalmente, el exceso de Cu puede ser secuestrado e inactivado por la acción de pequeños péptidos ricos en cisteínas que contribuyen a la quelación del metal en el citosol como las metalotioneínas (MT) y fitoquelatinas, estas últimas sintetizadas enzimáticamente a partir del glutatión por la acción de la fitoquelatinsintasa (Beck et al., 2003). Por último, existen sistemas de compartimentación que permiten retirar el exceso del metal del citosol. El principal compartimento para el almacenamiento de compuestos y sustancias tóxicas como el Cu en plantas es la vacuola. Se ha identificado una familia de proteínas que posee homología con la proteína humana MRP (del inglés, multi-drug resistance-associated protein) implicadas en la compartimentación de complejos metal-metalotioneína. El conocimiento de estos mecanismos de homeostasis está limitado a un número reducido de organismos. La mayoría han sido descritos en la levadura *Sacharomyces cerevisia*e, ampliamente utilizada como organismo modelo para su estudio, y en la planta *Arabidopsis thaliana* (Puig and Thiele, 2002; Williams and Mills, 2005; Pilon et al., 2006; Burkhead et al., 2009) (**Figura 8**).

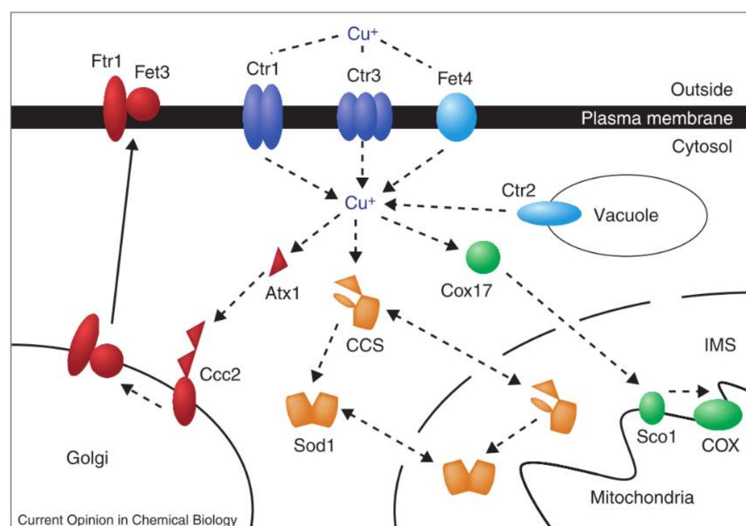


Figura 8. Mecanismos de transporte y distribución de Cu en la levadura modelo *S. cerevisiae* (Puig and Thiele, 2002).

2.3 Micorrizas y cobre

Los hongos MA, como se ha mencionado con anterioridad, contribuyen no sólo a la toma de macronutrientes (P y N) sino también de micronutrientes (Cu y Zn) (Smith & Read, 2008), mejorando enormemente la eficiencia de absorción de la planta hospedadora, en gran medida debido a la capacidad de exploración del micelio extraradical que puede llegar a sitios inaccesibles para las raíces. De hecho, se ha comprobado que la planta puede adquirir entre el 60-75% del Cu a través de la vía micorrícica (Li et al., 1991; Lee and George, 2005). Sin embargo, los conocimientos de los mecanismos de transporte de este micronutriente en la simbiosis son muy limitados. Tan solo, en plantas de *Medicago truncatula* se ha identificado un transportador de Cu de la familia CTR/COPT que se inducía fuertemente en raíces micorrizadas y que podría ser el responsable de la captación de Cu por la membrana periarbuscular (Gomez et al., 2009). Mientras que los sistemas de transporte de Cu en hongos MA aún no han sido descritos.

Por otro lado, cuando las concentraciones de los metales esenciales son supraóptimas, los hongos MA son capaces de lidiar con el exceso del metal e incrementar la tolerancia de la planta hospedadora frente a concentraciones tóxicas de metales. Varios estudios han demostrado la existencia de hongos MA tolerantes a metales, incluido el Cu, y cómo éstos son capaces de conferir una mayor tolerancia a la planta frente a este metal que otros hongos aislados de suelos no contaminados (Del Val et al., 1999; Liao et al., 2003). La mayor tolerancia de las plantas micorrizadas a niveles tóxicos de Cu se debe fundamentalmente a la capacidad de los hongos de inmovilizar el exceso del metal en las raíces y/o en el suelo mediante la acumulación del mismo en las estructuras que el hongo desarrolla en el interior y en el exterior de la raíz (Ferrol et al., 2009; Cornejo et al., 2013). Además, se ha demostrado que el desarrollo de la simbiosis MA potencia los mecanismos de tolerancia de la planta al exceso de Cu, tales como un incremento en la actividad de sistemas antioxidantes (Pallara et al., 2013), producción de metalotioneinas (Cicatelli et al., 2010) y fitoquelatinas (Merlos et al., 2016).

La conclusión general que se deriva de todos estos estudios es que los hongos MA contribuyen al establecimiento, desarrollo y supervivencia de las plantas tanto en suelos con problemas de deficiencia como de toxicidad de cobre. Estas observaciones han planteado el uso de los hongos MA en la biorremediación de suelos contaminados (Gohre and Paszkowski, 2006; Meier et al., 2012; Cabral et al., 2015) y en estrategias de biofortificación de cultivos (He and Nara, 2007; White and Broadley, 2009).

2.4 Mecanismos de homeostasis de Cu en los hongos MA

El conocimiento de los mecanismos de homeostasis de metales en los hongos MA resulta esencial, ya que además de sus inminentes consecuencias en el desarrollo del hongo, también afectan a la planta hospedadora a través del desarrollo de la simbiosis MA. Estos mecanismos de homeostasis están basados tanto en estrategias de secuestro y quelación del metal como en la regulación de los sistemas de transporte que determinan su adquisición y eflujo. Bajo condiciones de deficiencia de metales, deben activarse las proteínas transportadoras que median la adquisición del metal medio, mientras que bajo condiciones de toxicidad, los mecanismos descritos que tienen los hongos MA para lidiar con el exceso del metal están basados en estrategias de quelación y compartimentalización de metales en diferentes estructuras fúngicas (Ferrol et al., 2016), en la siguiente sección se detallan las descritas para el Cu.

2.4.1. Estrategias de supervivencia de los hongos MA bajo condiciones tóxicas de Cu

Los hongos MA utilizan diferentes estrategias para hacer frente a condiciones tóxicas de Cu en el suelo, entre éstas se han descrito estrategias basadas en la restricción de la entrada del metal, en la quelación intracelular y en su compartimentalización, además de la activación de sistemas antioxidantes y se detallan a continuación:

- Restricción de la entrada del metal: los hongos MA pueden evitar la entrada de Cu mediante la liberación de agentes con capacidad quelante al suelo o mediante su unión a estructuras de la superficie celular. Numerosos estudios avalan la capacidad de inmovilizar metales, incluido el Cu, de una glicoproteína producida por los hongos MA (Gonzalez-Chavez et al., 2004; Cornejo et al., 2008; Gil-Cardeza et al., 2014). Igualmente se ha descrito la unión del Cu a la pared de hifas y esporas, con mayor afinidad en estas últimas (González-Guerrero et al., 2008).
- Quelación intracelular: debido a la alta reactividad del Cu es especialmente importante la quelación del mismo una vez que ha alcanzado el citosol. Entre los ligandos capaces de llevar a cabo este secuestro e inactivación del Cu se encuentran aminoácidos y ácidos orgánicos, así como dos tipos de péptidos ricos en cisteínas que contribuyen a la quelación del metal en el citosol las metalotioneínas y las fitoquelatinas. Dos metalotioneínas han sido identificadas y caracterizadas en hogos MA, concretamente en *Gigaspora margarita* (Lanfranco et al., 2002) y en *R. irregularis* (González-Guerrero et al., 2007) siendo capaces de restablecer la tolerancia a Cu del mutante de *S. cerevisae* que carece de la metalotioneína *CUP1* y que es altamente sensible a concentraciones tóxicas de Cu (Ecker et al., 1986). Respecto a las fitoquelatinas, aunque tradicionalmente se pensaba que su producción estaba restringida a plantas, en la actualidad se sabe que su producción está extendida a más organismos. Shine y colaboradores (2015) pusieron de manifiesto la

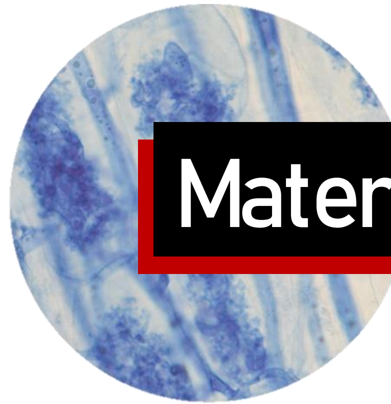
existencia de un gen en el genoma de *R. irregularis* que potencialmente codifica para una fitoquelatin-sintasa requerida para la síntesis de fitoquelatinas a partir de glutatión, aunque su contribución a la tolerancia de metales aún no ha sido descrita.

- Compartimentalización del metal: la acumulación de metales en vacuolas también ha demostrado ser un mecanismo importante de detoxificación en hongos MA. La presencia de Cu y otros metales como Zn y Cd en vacuolas fue demostrada por (González-Guerrero et al., 2008) en el hongo *R. irregularis* tras ser expuesto a concentraciones elevadas de estos metales. Debido a la co-localización de los metales con polímeros de poliP en vacuolas, se piensa que estos polímeros de poliP podrían contribuir a su estabilización. Por otro lado, se ha sugerido que el transportador tipo ABC identificado en *R. irregularis* Ri ABC podría contribuir a la internalización del Cu en las vacuolas (González-Guerrero et al., 2010). Además, los hongos MA pueden compartimentalizar el exceso de Cu en el interior de esporas que adquieren una coloración verde-azul debido a la internalización de este metal en el citoplasma. La presencia de esporas azules fue demostrada por (Cornejo et al., 2013) en *Claroideoglomus claroideum* en suelos contaminados con Cu y en *R. irregularis* crecido en cultivo monoxénico y expuesto a concentraciones elevadas de Cu.
- Eflujo del metal: Una forma alterativa de controlar los niveles intracelulares de metales es a través de su eflujo mediante proteínas transportadoras presentes en la membrana plasmática. Sin embargo, esta estrategia es más frecuente en procariotas que en eucariotas y hasta la fecha, solo se ha descrito un transportador de arsénico (As) en *R. irregularis* RiArsA que en colaboración con el transportador de captación de fósforo RiPT podría contribuir al eflujo de As (Gonzalez-Chavez Mdel et al., 2011).
- Defensas antioxidantes: como se ha comentado con anterioridad, el Cu a través de reacciones tipo Fenton genera especies reactivas de oxígeno que pueden causar daño en diferentes moléculas de importancia vital como el ADN, lípidos y proteínas (Linder, 1991) por lo que la activación de defensas antioxidantes también resulta de gran importancia para hacer frente a concentraciones tóxicas de este metal. En este sentido cabe destacar la identificación de una serie de enzimas que contribuyen a la detoxificación de especies reactivas en hongos MA, como RiSOD1 una superóxido dismutasa (Gonzalez-Guerrero et al., 2010), RiGRX1 una glutarredoxina con actividad oxidorreductasa, peroxidasa y glutatión transferasa (Benabdellah et al., 2009b) y RiPDX1 una enzima implicada en la biosíntesis de vitamina B6 en *R. irregularis* (Benabdellah et al., 2009a).

Objetivos

El objetivo general de la tesis doctoral es profundizar en el estudio de los mecanismos de homeostasis de cobre en la simbiosis MA mediante la caracterización de los sistemas de transporte de este micronutriente en la simbiosis y de su repercusión sobre la homeostasis de Cu y el desarrollo de la planta hospedadora. Para la consecución de este objetivo se plantearon los siguientes objetivos específicos:

1. Identificar las diferentes familias de transportadores de Cu en el hongo MA *R. irregularis* (Capítulo I).
2. Analizar el papel de los transportadores de influjo e eflujo de Cu de *R. irregularis* en la homeostasis de Cu (Capítulos II y III).
3. Analizar la posible contribución de las P_{1B}-ATPasas de metales de la planta hospedadora a la tolerancia de plantas micorrizadas de maiz a altos niveles de Cu (Capítulo IV).



Materiales y Métodos

1. Material biológico y condiciones de crecimiento

El hongo micorríco arbuscular utilizado en el presente estudio fue el aislado DAOM 197198 de *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler, actualmente renombrado como *Rhizoglomus irregulare* (Błaszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl (Sieverding et al., 2015). Puesto que esta nomenclatura es relativamente reciente y aún no está muy establecida, por simplificar en esta Tesis Doctoral se ha continuado con la nomenclatura tradicional. La elección se basa en que es uno de los hongos MA que mejor se cultiva *in vitro* y su genoma ha sido secuenciado y se encuentra disponible en el portal de libre acceso JGI Joint Genome Institute (https://genome.jgi.doe.gov/portal/). Actualmente hay incluso varias versiones de su genoma, la primera versión (v1.0) fue depositada por Tisserant y colaboradores (2013) y recientemente se ha publicado la segunda versión (v2.0) incluyendo la de varios aislados de *R. irregularis* A1, A4, A5, B3 y C2 (v1.0) procedentes de una misma población en Suiza (Chen et al., 2018).

El inóculo fúngico usado tanto para su cultivo *in vitro* establecido en cultivo monoxénico como para su cultivo *in vivo* en un sistema tipo sándwich recientemente descrito por Pepe y colaboradores (2017) y que posteriormente se detallarán, consistió en una mezcla de esporas, micelio y fragmentos de raíces micorrizadas obtenida en cultivo monoxénico.

La planta hospedadora utilizada fue *Zea mays* L., uno de los cereales con mayor importancia agrícola y cuyo crecimiento se ve afectado por la presencia de Cu y por el desarrollo de MA, como se ha descrito en varios trabajos (Ouzounidou et al., 1995; Weissenhorn et al., 1995; Keltjens and van Beusichem, 1998; Liu et al., 2000; Madejón et al., 2009; Gerlach et al., 2015; Merlos et al., 2016; Ramirez-Flores et al., 2017). Concretamente se ha utilizado la variedad Orense por ser especialmente sensible al Cu (Madejón et al., 2009). Las semillas fueron suministradas por Semillas Fitó, Barcelona, España.

Para estos ensayos en planta, el inóculo fúngico se administró en sustrato sepiolita: vermiculita (1:1, v:v) que contenía una mezcla de esporas, micelio y fragmentos de raíces micorrizadas procedentes de cultivos trampa establecidos con *Trifolium repens* L. y *Sorghum vulgare* L. La proporción usada para la inoculación de las plantas fue del 10% sobre el volumen final del sustrato de crecimiento utilizado.

1.1 Cultivo monoxénico

Dado el carácter de simbiontes obligados de los hongos MA y con el fin de obtener material fúngico libre de material vegetal y de otros microorganismos, se establecieron cultivos *in vitro* monoxénicos de *R. irregularis* en asociación con raíces transformadas (Ri ADN-T) clon DC2 de zanahoria (*Daucus carota* L.) en placas Petri bi-compartimentadas según (St-Arnaud et

al., 1996), con algunas modificaciones. Brevemente, ambos compartimentos se rellenaron con medio sólido M (Chabot et al., 1992) y en uno de ellos se puso el inóculo fúngico consistente en esporas, micelio y fragmentos de raíces micorrizadas y 2-3 fragmentos de raíces no micorrizadas. Las placas se incubaron en oscuridad a 24 °C y se mantuvieron alrededor de 6 - 8 semanas hasta que el otro compartimento estaba ampliamente colonizado por el micelio extraradical del hongo (ERM) y algunas raíces, quedando definido como compartimento de la raíz. El compartimento viejo fue desechado y se rellenó con medio líquido M modificado sin la fuente carbonada sacarosa y con el doble de concentración en el resto de sus componentes menos de KH_2PO_4 (medio M-C) que tras 2-3 semanas fue colonizado por el hongo, quedando definido como compartimento de las hifas (**Figura 9**). En este estadio, dependiendo del uso final del cultivo, el contenido de este compartimento se mantiene tal cual (condiciones control) o se reemplaza por medio líquido M-C nuevo con modificaciones en su composición determinadas por los tratamientos aplicados que se detallarán en los Capítulos correspondientes. El ERM se aisló del compartimento de las hifas con pinzas, con cuidado de retirar cualquier resto de material vegetal, se secó con papel de filtro, se congeló en N líquido y se conservó -80 °C hasta su uso. Por otro lado, las raíces micorrizadas provistas de micelio intraradical (IRM) se aislaron del compartimento de la raíz con pinzas, se secaron con papel de filtro, se congelaron en N líquido y se guardaron a -80 °C hasta su uso, excepto una alícuota que guardó a 4 °C para estimar la colonización micorrícica arbuscular de las raíces.

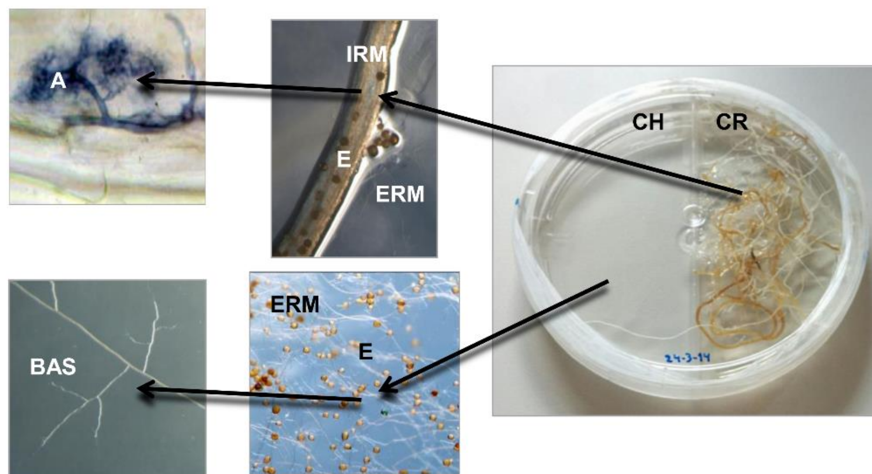


Figura 9. Cultivo *in vitro* de *Rhizophagus irregularis*. Cultivos monoxénicos de *R. irregularis* establecidos en asociación con raíces transformadas (Ri ADN-T) de *Daucus carota* en placas Petri bi-compartimentadas (St-Arnaud et al., 1996). CH: compartimento de las hifas; CR: compartimento de la raíz; A: arbúsculo; BAS: “Branched Absorbing Structures”; E: esporas; ERM: micelio extraradial, abreviatura del inglés “extraradical mycelium”; IRM: micelio intraradical, abreviatura del inglés “intraradical mycelium”.

Para los estudios de comparación de la expresión génica entre ERM *vs.* IRM, el IRM se aisló de una forma alternativa. Se colocaron varios fragmentos de raíz no micorrizada sobre un compartimento que contenía medio sólido M densamente colonizado por el micelio externo del hongo y se mantuvieron durante 15 días en oscuridad a 24 °C. Tras este tiempo, las raíces fueron colectadas bajo una lupa binocular retirando con cuidado las hifas extraradicales adheridas a las mismas. Igual que anteriormente, se secaron en papel de filtro, se congelaron en N líquido y se conservaron a -80 °C hasta su uso, excepto una alícuota que se guardó a 4 °C para estimar la colonización micorrícica arbuscular.

1.2 Sistema Sándwich

Adicionalmente, con el fin de cultivar al hongo en un sistema *in vivo* pero que permitiese igualmente obtener micelio extraradical libre de material vegetal, se utilizó un sistema de cultivo tipo sándwich denominado "sistema experimental bidimensional de toda la planta" descrito por Pepe y colaboradores (2017), con algunas modificaciones (**Figura 10**). Brevemente, se esterilizaron en superficie semillas de Achicoria (*Cichorium intybus* L.) y se germinaron durante 10-15 días sobre arena estéril. Las plántulas se trasplantaron a macetas de 50 mL que contenían arena estéril y se inocularon con esporas, ERM y fragmentos de raíces micorrizadas procedentes de cultivo monoxénico. Estas macetas se confinaron en bolsas transparentes (Sigma-Aldrich, B7026) y se mantuvieron durante un mes en cámara de cultivo a 24 ºC/ 21 ºC día/ noche con un fotoperiodo de 16 h de luz y 8 h de oscuridad. Tras este tiempo, las raíces se limpiaron, se envolvieron en una malla de nailon con un diámetro de poro de 41µM (Millipore NY4100010), se dispusieron entre dos membranas de esteres mezclados de celulosa de 13 cm con un diámetro de poro de 0.45 µm (MF-Millipore HAWP14250) a modo de sándwich en placas Petri de 14 cm de diámetro rellenas de arena estéril y a las que se les había hecho un agujero en uno de los lados para dejar salir la parte aérea de la planta. Las placas se sellaron con Parafilm, se envolvieron en aluminio, se confinaron en bolsas transparentes y se mantuvieron en cámara de cultivo con las mismas condiciones de crecimiento que anteriormente. Las plantas se regaron semanalmente con solución nutritiva de Hoagland 0.5X modificada que contenía 125µM de KH_2PO_4 y 0.16 µM de $CuSO_4$ (condiciones control) o sin Cu (tratamiento de deficiencia) como se detallará en el Capítulo II. Transcurridas dos semanas en este último sistema las plantas se cosecharon. Las raíces quedaron atrapadas en la malla de nailon mientras que el ERM la atravesó, quedando extendido sobre la superficie de las membranas de las que se extrajo con pinzas. Tanto el ERM como las raíces, se secaron con papel de filtro, fueron inmediatamente congelados en N líquido y se guardaron a -80 ºC hasta su uso, excepto una alícuota de raíces por planta que se guardó a 4 °C para estimar la colonización micorrícica arbuscular.

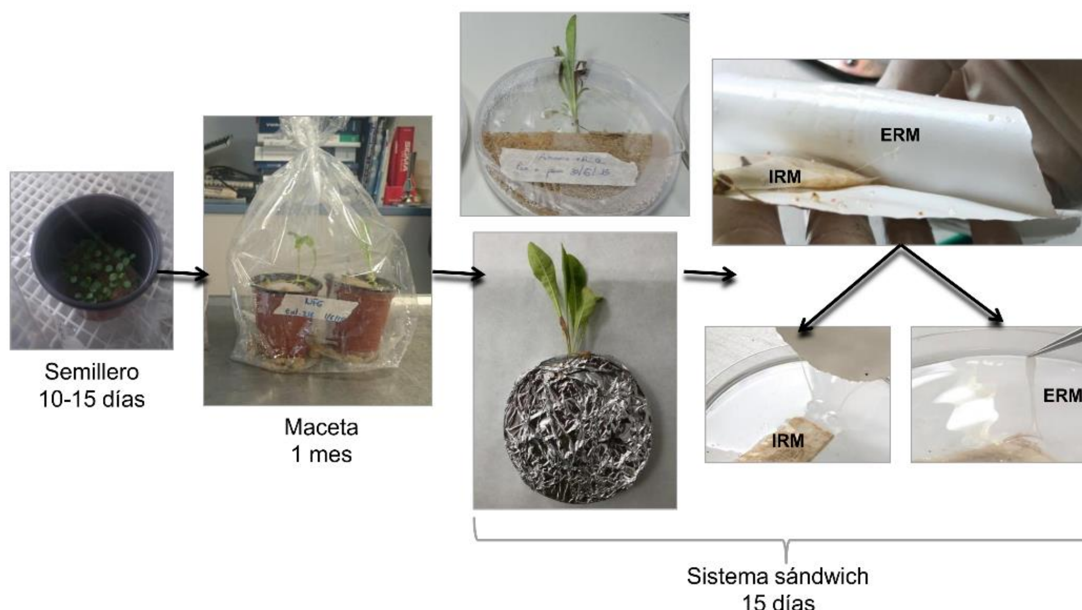


Figure 10. Cultivo *in vivo* de *Rhizophagus irregularis*. Sistema sándwich basado en Pepe *et al.* (2017) establecido con *R. irregularis* y plantas de *Cichorium intybus*. ERM: micelio extraradial, abreviatura del inglés "extraradical mycelium"; IRM: micelio intraradical, abreviatura del inglés "intraradical mycelium".

1.3 Ensayos en planta

Los ensayos establecidos para el estudio de los genes por parte de la planta hospedadora se realizaron con plantas de maíz *Z. mays* variedad Oropesa que es especialmente sensible al Cu, como ya se ha comentado con anterioridad.

Las semillas se esterilizaron en superficie y se pusieron a germinar sobre discos de papel de filtro humedecidos con agua estéril en placas Petri durante 3 días a 28 ºC en oscuridad, tras los cuales se trasplantaron a macetas de 1L que contenían el sustrato tratado o no con diferentes concentraciones de Cu como se detallará en el capítulo IV. Para la inoculación de las plantas con *R. irregularis* se añadió 10% del inóculo fúngico en sustrato sepiolita: vermiculita (1:1, v:v) mezclado manualmente con el sustrato correspondiente (plantas micorrizadas) mientras que las no inoculadas recibieron un filtrado del inóculo fúngico para abastecerlas de una microbiota similar pero exenta de hongos MA (plantas control). Las plantas se mantuvieron en cámara de cultivo a 25 ºC / 18 ºC día/ noche con un fotoperiodo de 16 h de luz y 8 h de oscuridad. La cosecha se realizó a las 5 semanas tras la siembra. La parte aérea y raíz de las plantas se secaron con papel de filtro, se congelaron en N líquido y se guardaron a -80 ºC hasta su uso, excepto una alícuota de raíces por planta que se guardó a 4 °C para estimar la colonización micorrícica arbuscular.

2. Cuantificación de MA

La colonización micorrícica de las raíces se estimó tras su digestión con KOH al 10% y tinción con azul tripán al 0.05% (Phillips and Hayman, 1970) siguiendo el método de intersección de (Giovannetti and Mosse, 1980) bajo una lupa binocular o el método de Trouvelot et al. 1986 bajo un microscopio óptico y usando el programa Mycoclac (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) para el cálculo de los diferentes parámetros de micorrización.

La colonización micorrícica también se cuantificó por métodos moleculares mediante el análisis de la expresión génica del factor de elongación 1α de *R. irregularis* (*RiEF1α*; Nº GenBank DQ282611) normalizado con el factor de elongación 1α de la planta hospedadora correspondiente.

3. Técnicas moleculares

3.1 Extracción de ácidos nucleicos y síntesis de ADNc

La extracción de ARN genómico de *R. irregularis* se realizó a partir de ERM desarrollado en el del compartimento de las hifas de placas control usando "DNeasy Plant Mini Kit" (Qiagen) de acuerdo con las instrucciones del fabricante.

La extracción de ARN total del ERM y de las raíces micorrizadas de zanahoria procedentes de cultivo monoxénico se realizó con "Plant RNeasy Kit" (Qiagen) siguiendo las indicaciones del fabricante. El ARN total de las raíces de achicoria micorrizadas procedentes del sistema sándwich, así como de la parte aérea y de las raíces de los ensayos en planta establecidos con maíz, se extrajo usando el método de extracción fenol/SDS seguido de su precipitación con LiCl (Kay et al., 1987).

Los ARNs se trataron con DNasa usando "RNA-free DNase set" (Qiagen) de acuerdo con las instrucciones del fabricante. Posteriormente se cuantificaron usando el "Nanodrop 1000 Spectrophotometer" (Thermo Scientific). La síntesis de ADNc se realizó a partir de 1 µg de ARN total tratado con DNasa en un volumen final de 20 µL empleando 200 U de la retrotranscriptasa "SuperScript III RT" (Invitrogen) y 2.5 µM de "oligo $(dT)_{20}$" (Invitrogen) de acuerdo con las indicaciones del fabricante.

3.2 Análisis de la expresión génica

La expresión génica se analizó mediante PCR cuantitativa a tiempo real en el equipo "iQ™5 Multicolor Real-Time PCR Detection System" (Bio-Rad). Las reacciones se realizaron en un volumen final de 20 µL con 1 µL de una dilución 1:10 de ADNc, 200 nM de cada oligo y 10 µL de "iQ™ SYBR Green Supermix" (Bio-Rad). El programa de PCR consistió en una incubación inicial a 95 ºC durante 3 min, seguido de 38 ciclos a 95 °C durante 30 segundos, 58 ºC- 60 ºC durante 30 s (58 ºC para los genes del hongo y 60 ºC para los genes de la planta) y 72 ºC durante 30 segundos. La fluorescencia emitida por el "SYBR Green" se midió al final de la extensión de cada ciclo. Seguidamente se realizó una curva de disociación para asegurar la especificidad del amplicón en cada reacción. Antes de proceder a analizar la expresión génica, se comprobó que la eficiencia de los oligonucleótidos diseñados se encontrase entre el 90 -105%. Igualmente, se comprobó que los oligos diseñados para el hongo no amplificasen en la planta hospedadora correspondiente y viceversa.

Todas las determinaciones en PCR cuantitativa a tiempo real se realizaron en al menos tres muestras biológicas independientes con el ciclo umbral (Ct) en duplicado, en al menos dos experimentos de PCR independientes. La abundancia relativa de los transcritos se calculó mediante el método $2^{-\Delta\Delta CT}$ establecido por (Livak and Schmittgen, 2001) normalizado con el factor de elongación 1α de *R. irregularis* (*RiEF1α*; GenBank N° DQ282611) o con el factor de elongación 1α de *Z. mays* (*ZmEF1α;* GenBank N° NM_001112117) según se tratasen de genes del hongo o del maíz, respectivamente.

3.3 Aislamiento y clonación de los genes de interés

Las secuencias de ADNc de los transportadores de Cu del hongo se verificaron y completaron cuando fue necesario mediante la técnica de 5´/3' RACE "Rapid Amplification of cDNA Ends" usando "SMARTer® RACE 5'/3' Kit" (Clontech) siguiendo el protocolo especificado por el fabricante. El clon genómico y de la secuencia ADNc de los genes en estudio se obtuvieron mediante la amplificación por PCR a tiempo final de ADN genómico o ADNc de *R. irregularis*, respectivamente, aislado a partir de ERM obtenido en cultivo monoxénico bajo condiciones control usando una pareja de oligos que flanquease la secuencia completa del gen correspondiente. Los productos de PCR se clonaron en el vector pGEM-T Easy (Promega) siguiendo las indicaciones del fabricante y seguidamente se usaron para transformar *Escherichia coli* DH5α quimiocompetentes según protocolos standard. La extracción del ADN plasmídico se realizó con "GenElute™ Plasmid Miniprep Kit" (Sigma-Aldrich) y finalmente las secuencias de ADN clonadas se verificaron mediante secuenciación ("ABI PRISM 3130*xl* Genetic Analyzer", Applied Biosystems).

3.4 Hibridación *in situ*

Los experimentos de hibridación *in situ* se llevaron a cabo en colaboración con Pierre Berthomieu y Geneviève Conéjéro, del B&PMP, INRA (Montpellier, Francia), según lo establecido por (Jabnoune et al., 2009), con algunas modificaciones tal y como se detalla en el Capítulo III de esta Tesis Doctoral. Brevemente, se generaron sondas sentido y antisentido específicas para el gen en estudio mediante PCR incorporando una secuencia promotora de la T7 RNA polimerasa. Los productos de PCR se purificaron y se trascribieron a ARN mediante el kit comercial "MAXIscript T7 Transcription Kit" (Invitrogen) según las condiciones establecidas por el fabricante, proceso en el que se incorporan uracilos marcados con digoxigenina. El marcaje de las sondas con digoxigenina se verificó mediante Dot-Blot.

Por otro lado, fragmentos de 3 mm de raíces micorrizadas y no minorizadas de tomate se fijaron con 4 % paraformaldehido (p/v), 0,1 % Triton X-100 (v/v) en tampón salino PBS durante toda la noche a 4 ºC, aplicando previamente dos periodos cortos de 5 minutos de vacío. Tras su lavado con tampón PBS (3 veces, 30 minutos), las raíces fueron deshidratadas aplicando series de etanol-butanol y embebidas en parafina (ParaplastPlus, Leica BioSystems) a 56ºC, tras lo cual se realizaron cortes de 8 µm en el micrótomo Leica RM2255 tanto transversales como longitudinales y se montaron sobre portaobjetos silanizados (Euromedex). Sobre estos cortes se realizó la hibridación con 600 µg de sonda, previamente tratados con proteinasa k. La detección de las sondas marcadas con digoxigenina se realizó con un anticuerpo Anti-digoxigenina acoplado a la fosfatasa alcalina (Roche), empleando un sustrato para esta enzima no fluorescente denominado Vector Blue según las condiciones establecidas por el fabricante ("Vector Blue Alkaline Phosphatase Substrate Kit", Vector Laboratories). Finalmente, los resultados se visualizaron usando el microscopio Nikon Eclipse Ni-E.

4. Sistemas de expresión heteróloga en levadura

Una información más detallada sobre el uso de sistemas heterólogos aplicados al estudio de los hongos MA se podrá encontrar en el capítulo "Functional analyses of arbuscular mycorrhizal fungal genes in yeast" del libro "Arbuscular Mycorrhizal fungi: Methods and Protocols" que incluye una recopilación de los principales protocolos empleados en el estudio de los hongos MA, en el que he participado recientemente y que se encuentra en fase de publicación.

4.1 Caracterización funcional

La caracterización funcional de los genes del hongo se realizó en un sistema heterólogo utilizando cepas mutantes de *S. cerevisiae* deficientes en una función de interés. Con este fin, los genes fueron clonados en el vector de expresión en levadura pDR196. Para ello fue necesario incluir por PCR dianas de enzimas de restricción que flanqueasen el ADNc. El proceso de

clonación y las enzimas de restricción seleccionadas para cada gen se detallan en los Capítulos correspondientes.

Los mutantes de *S. cerevisiae* se transformaron con las construcciones correspondientes para cada gen y el vector vacío (control negativo) empleando el método de Gietz and Schiestl (2007). Los transformantes positivos se seleccionaron por auxotrofía a uracilo. Tras comprobar las construcciones por secuenciación, se llevaron a cabo los ensayos de complementación en el medio selectivo correspondiente para cada cepa, analizándose la capacidad del gen en estudio de revertir el fenotipo mutante.

Las distintas cepas mutantes de *S. cerevisiae* se crecieron en medio rico YPD ("Yeast extract Peptone Dextrose") y una vez transformadas, en medio SD ("Syntetic Dextrose") sin uracilo para mantener la presión selectiva sobre el plásmido.

4.2 Localización subcelular

La localización subcelular de las proteínas del hongo fue realizada mediante la fusión N o C terminal del gen de interés correspondiente a eGFP ("enhanced Green Fluorescence Protein") mediante su clonación en el vector pFGWDR196 o pGWFDR196, respectivamente, provistos de tecnología Gateway (Invitrogen). Una vez transformadas las cepas mutantes de *S. cerevisiae* con los plásmidos correspondientes, se comprobó que la fusión a GFP no interfería en la funcionalidad de la proteína a través de ensayos de complementación. La señal de fluorescencia emitida por la GFP se detectó en cultivos de levadura en fase exponencial en el microscopio confocal Leica TCS SP8 usando una longitud de onda de excitación de 488 nm. Para reducir problemas de sobreexpresión, las levaduras fueron tratadas con el inhibidor de síntesis proteica cicloheximida (100 µM) durante 45 minutos justo antes de su visualización. Las imágenes fueron procesadas con el programa ImageJ.

Estos experimentos de localización subcelular de las proteínas del hongo en levadura se realizaron en colaboración con Pierre Berthomieu y Carine Alcon del B&PMP, INRA (Montpellier, Francia).

5. Métodos bioinformáticos

5.1 Identificación de genes de interés

La identificación de genes codificantes para transportadores de Cu del hongo MA *R. irregularis* y de la planta *Z. mays* se realizaron mediante el uso de la base de datos del "Joint Genome Institut" (JGI, https://genome.jgi.doe.gov/portal/) en la que se encuentran depositadas dos versiones del genoma del hongo *R. irregularis* DAOM 197198 (Tisserant et al., 2013; Morin et al., 2019) y de la base de datos de proteínas de membrana de plantas Aramemnon

(http://aramemnon.uni-koeln.de/), respectivamente, tal y como se especifica en los Capítulos correspondientes. Las secuencias identificadas en estas bases de datos, junto con secuencias ortólogas de otros organismos, se utilizaron para realizar búsquedas adicionales a través de la herramienta BLAST ("Basic Local Alignment Search Tool") disponible en la página web del NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

5.2 Manejo de secuencias

El manejo, ensamblaje y alineamiento de las secuencias obtenidas en las bases de datos y durante el proceso de clonación, se realizaron mediante el uso del paquete bioinformático DNAstar de Lasergene v.7.0.

5.3 Análisis *in silico* de secuencias

El análisis de las secuencias promotoras de los genes se realizó mediante el uso de las herramientas disponibles en la base de datos de promotores de *S. cerevisiae* (SCPD, http://rulai.cshl.edu/SCPD/) mientras que la búsqueda de elementos *cis* reguladores específicos se realizó mediante el análisis de coincidencia de patrones de ADN disponible en el servidor RSAT de hongos (http://rsat-tagc.univ-mrs.fr/rsat/).

Los porcentajes de identidad y similitud entre proteínas se calcularon mediante la herramienta "Ident and Sim" disponible en el programa "Sequence Manipulation Suite" v.2.0 (http://www.bioinformatics.org/sms2/ident_sim.html). Las predicciones sobre la presencia de dominios transmembrana se realizaron indistintamente con los algoritmos TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), SOSUI v. 1.11 (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) y TOPCONS (http://topcons.cbr.su.se/) y los modelos estructurales se generaron con la herramienta MyDomains de Prosite (https://prosite.expasy.org/mydomains/). Finalmente, las predicciones de localización subcelular se realizaron considerando los resultados obtenidos en los servidores WoLF PSORT I / II (https://wolfpsort.hgc.jp/; https://www.genscript.com/wolf-psort.html) y TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/).

Las secuencias de aminoácidos identificadas en las bases de datos se alinearon con las ortólogas de otros organismos utilizando Clustal Omega (https: // www. ebi.ac.uk/Tools/msa/clustalo/) y fueron importadas al programa MEGA v.6. para la realización de análisis filogenéticos. Los árboles filogenéticos se construyeron en base al método "Neighbor-Joining", con 1000 repeticiones bootstrap, utilizando el modelo de Poisson y la opción "pairwise deletion of gaps" para el cálculo de las distancias evolutivas.

6. Análisis estadístico

Todos los análisis estadísticos se llevaron a cabo con el programa IBM SPSS Statistic v.23. En general, se utilizó la prueba T-Student para comparaciones entre dos medias o ANOVA de una o dos vías seguido de diferentes pruebas post-hoc para establecer comparaciones múltiples entre más de dos medias, según los objetivos y diseño experimental de cada Capítulo. Las diferencias con un valor de $P < 0.05$ se consideraron significativas.

Una metodología más detallada de los protocolos empleados se puede consultar en el apartado correspondiente a materiales y métodos de cada Capítulo.

Resultados

CHAPTER I: Genome-wide análisis of copper transporters in the arbuscular mycorrhizal fungus *Rhizophagus irregularis*

Adapted from:

Tamayo, E., Gómez-Gallego, T., Azcón-Aguilar, C., Ferrol, N. 2014.

Genome-wide analysis of copper, iron and zinc transporters in the arbuscular mycorrhizal fungus *Rhizophagus irregularis.*

Frontiers in Plant Science 5. doi: 10.3389/fpls.2014.00547.

Introduction

The transition metals such as Cu play essential and catalytic roles throughout the cell in various subcellular compartments. These metal cofactors are critical for processes such as transcription, translation, the production of ATP in the mitochondria and the scavenging of toxic free radicals (van Ho et al., 2002; Schaible and Kaufmann, 2004; Kim et al., 2008). However, these metals are a highly reactive group of elements and are toxic at high concentrations (Valko et al., 2005). Therefore, their biological concentrations are finely regulated in living cells. To maintain micronutrient homeostasis, all organisms have developed a complex network of metal uptake, chelation, trafficking and storage processes (Festa and Thiele, 2011). Transporters represent the first line of defense to perturbations of cellular and subcellular metal homeostasis and constitute an important component of this network. When metal reserves are depleted, transporters contribute to the specific supply and distribution of the needed cofactor before deficiency symptoms appear. However, when the concentration of metal within the cell exceeds the cell's buffering capacity, transporters provide the route to expel excess cofactors before toxicity occurs (Nies, 2007). The toxic heavy metals, such as cadmium, lead, mercury, and nickel, have no physiological function but compete with the transporters of the essential biological metals. Therefore, the activity and specificity of the transporters of physiologically important heavy metals also control the lethality of the toxic metals.

Arbuscular mycorrhizal (AM) fungi are soil microorganisms that establish symbiotic mutualistic associations with most land plants. These fungi provide their host plants an efficient supply of low mobility mineral nutrients, mainly phosphorus and some micronutrients such as Cu and Zn. Thanks to the hyphal network they develop in the soil, AMF acquire nutrients not only for their own needs, but also for delivering them to the host plant. In return, the plant supplies the fungus with carbon compounds (Smith and Read, 2008). Besides improving plant mineral nutrition, AMF can alleviate heavy metal toxicity to their host plants (Göhre and Paszkowski, 2006; Lingua et al., 2008). Heavy metal tolerant AMF ecotypes have been isolated from polluted soils and these indigenous populations cope better with heavy metal toxicity than those isolated from unpolluted soils (del Val et al., 1999). To persist in environments with high heavy metal content, AMF have evolved a series of strategies to avoid the damage produced by the metal, such as compartmentalization of the metal excess in some spores (González-Guerrero et al., 2008; Cornejo et al., 2013) and highly efficient homeostatic mechanisms (Ferrol et al., 2009; González-Guerrero et al., 2009). Despite the central role of metal transporters in heavy metal homeostasis, only a gene encoding a Zn transporter has been characterized in AMF to date (González-Guerrero et al., 2005).

With the genome of *Rhizophagus irregularis* available (Tisserant et al., 2013), we have the unique opportunity to identify and present a global view of proteins involved in heavy metal transport in an AM fungus. In this work we have taken advantage of the recently released genome sequence of *R. irregularis* to establish a repertoire of candidate genes potentially involved in the transport of Cu in this fungus and to interpret them in light of its extremely adaptable character to grow in conditions of heavy metal deficiency or toxicity. This *R. irregularis* repertoire has been compared with that present in some reference fungi. To get some clues about the expression profiles of these genes throughout the fungal life cycle, we explored the published transcriptomic profiles in the extraradical mycelium (ERM) and symbiotic roots (intraradical mycelium, IRM) obtained using *the R. irregularis* expression oligoarray (Tisserant et al., 2012) and the RNA-Seq reads obtained from germinated spores and Medicago-colonized roots (Tisserant et al., 2013).

Materials and Methods

Gene identification

Amino acid sequences of fungal Cu transporters were retrieved from the freely accessible transport databases TCDB (http://www.tcdb.org/) and TransportDB (http://www.membranetransport.org/). These sequences were used to search for orthologous sequences in the filtered model dataset of *R. irregularis* on the JGI website (https://genome.jgi.doe.gov/Gloin1) via a protein BLAST. A second search was performed via a keyword search directly.

Since many of the fungal reference proteins were phylogenetically distant from *R. irregularis*, manually curated *Laccaria bicolor* (https://genome.jgi.doe.gov/Lacbi2), *Tuber melanosporum* (https://genome.jgi.doe.gov/Tubme1) and *Rhizopus oryzae* (https://genome.jgi.doe.gov/Rhior3) databases were used to look for additional orthologous sequences in the filtered model dataset of *R. irregularis*. This was also done via a BLASTp, run with the standard program settings.

Sequences analyses

Searches for conserved domains in the orthologous proteins found in *R. irregularis* were performed using the Conserved Domain Database at NCBI. (https://www.ncbi.nlm.nih.gov/cdd). Predictions of putative transmembrane domains were made using the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SMART software (http://smart.embl-heidelberg.de/). Full-length amino acid sequences were aligned with the orthologous sequences of a number of fungi representatives of distinct taxonomic groups by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Alignments were imported into the Molecular

Evolutionary Analysis (MEGA) package version 6. Phylogenetic analyses were performed using the Neighbor-Joining (NJ) method implemented in MEGA using the Poisson correction model and pairwise deletion of gaps option for distance computation. Bootstrap analyses were carried out with 1000 replicates.

Results and Discussion

The release of the *R. irregularis* genome (Tisserant et al., 2013; Lin et al., 2014) allowed making a genome-wide inventory of genes coding for Cu transporters. This *in silico* analysis allowed identification of seven open reading frames in the *R. irregularis* genome, which potentially encode Cu transporters, belonging to two multigene families (**Table 1**).

Table 1. Overview of the putative Cu transporters identified in the *Rhizophagus irregularis* genome. Columns 1 to 5 contain protein name, protein JGI identification (JGI ID) number, ratio of expression levels in *M. truncatula* symbiotic roots (IRM) to 2 d germinated spores (calculated from the RNA-seq reads in Tisserant et al., 2013), and ratio of expression levels in IRM to ERM (from Tisserant et al., 2012). CTR, Cu transporter. Modified from Tamayo et al. (2014).

Protein name	JGI ID	Ratio IRM/spore	Ratio IRM/ERM
CTR family			
CTR1	153709	0.5	-
CTR2	335281	5	2.9
CTR3	67076	0.3	-
P_{1B}-ATPase family			
CCC2.1	335789	2.6	2.6
CCC2.2	83433	1	-
CCC2.3	236684	0.7	-
CRD1	32309	1	-

Despite the long history of Cu as fungicide, AM fungi are able to grow and persist in Cu contaminated soils. The morphological alterations observed in the ERM of *R. irregularis* grown *in vitro* in association with root organ cultures in media without Cu or with Cu concentrations that are lethal to a majority of other organisms reflect its extremely adaptable character (**Figure 1**). Several studies have shown that AMF finely regulate the cytosolic Cu levels when confronted to excess Cu (González-Guerrero et al., 2008) and that the fungus responds to Cu toxicity by inactivating the excess of Cu in the cytosol through the activity of metallothioneins and the activation of antioxidant defenses (for a review see Ferrol et al., 2009). However, nothing is known about the Cu transporters that move Cu across the *R. irregularis* membranes. The two major families of Cu transporters identified in the *R. irregularis* genome are described below.

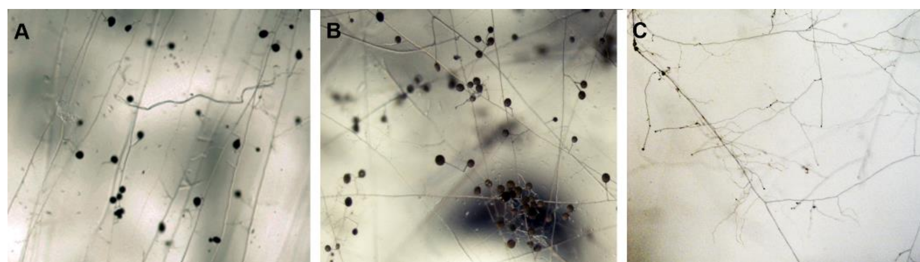


Figure 1. Effect of Cu on extraradical hyphal development of *Rhizophagus irregularis* grown in vitro in association with root organ cultures. Extraradical mycelium (ERM) was grown in minimal medium lacking Cu (A), containing 0.5 µM Cu (B), or 500 µM Cu (C) Mycelial architecture was altered markedly when the fungus developed in a Cu-free media (A) and in the presence of 500 µM Cu (C).

The copper transporter (CTR) family

Our *in silico* analysis revealed that *R. irregularis* likely acquires Cu through the activity of a transporter belonging to the CTR family of Cu transport proteins. This protein family is highly conserved across all fungal species and mediates Cu transport into the cytoplasm. CTR proteins are small integral membrane proteins that contain three transmembrane domains, with the N-terminus located in the extracellular space and the C-terminus in the cytosol. A series of clustered methionine residues in the hydrophilic extracellular domain, and a MXXXM motif in the second transmembrane domain, are important for Cu uptake. These methionine residues probably coordinate Cu during the process of metal transport (Yuan et al., 2011).

In *Saccharomyces cerevisiae*, Cu is transported into the cytosol by three high-affinity transporters (CTR1, CTR2, and CTR3). While CTR1 and CTR3 are located in the plasma membrane, and acquire Cu from the environment (Dancis et al., 1994; Peña et al., 2000), CTR2 is found in the tonoplast and pumps Cu into the cytosol (Portnoy et al., 2001; Puig and Thiele, 2002). The *R. irregularis* genome also contains three genes putatively encoding CTRs. The predicted genes and proteins have been named according to their orthologs in *S. cerevisiae*. These proteins clustered into two different clades in a phylogenetic Neighbor- Joining tree. RiCTR1 and RiCTR3 are more closely related to the *S. cerevisiae* plasma membrane CTR proteins, while RiCTR2 is highly homologous to the fungal vacuolar CTR2 transporters (**Figure 2**).

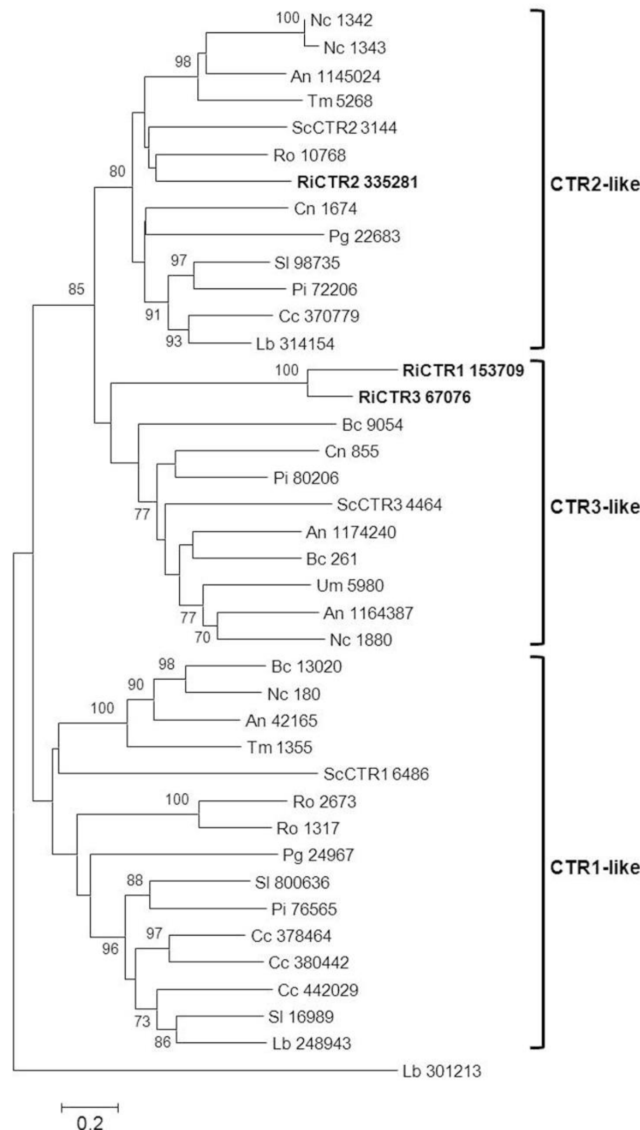


Figure 2. Phylogenetic relationships of the *Rhizophagus irregularis* copper transporters (CTR) with homologous sequences from selected species representative of the major fungal phyla. The Neighbor-Joining tree was created with MEGA 6. Protein JGI identification numbers are indicated. *R. irregularis* genes are shown in bold. Organisms: An, *Aspergillus niger*; Bc, *Botrytis cinerea*; Cc, *Coprinopsis cinerea*; Cn, *Cryptococcus neoformans*; Lb, *Laccaria bicolor*; Nc, *Neurospora crassa*; Pi, *Piriformospora indica*; Pg, *Puccinia graminis*; Sc, *Saccharomyces cerevisiae*; Sl, *Suillus luteus*; Tb,*Tuber melanosporum*; Ri, *Rhizophagus irregularis*; Ro, *Rhizopus oryzae*; Um, *Ustilago maydis*. Bootstrap values above 70 and supporting a node used to define a cluster are indicated.

Since CTR proteins are highly specific for reduced Cu^{+} and Cu widely exists as Cu^{2+}, transport by CTR is dependent on reduction of Cu by a ferric/cupric reductase (Hassett and Kosman, 1995). Orthologous sequences of the fungal cell surface Cu metalloreductases encoded by the FRE genes are present in the *R. irregularis* genome, suggesting that the Cu reduction process is similar to that described for other fungi. Upon entering the cytoplasm, small molecules and proteins sequester the Cu ions, and the resulting concentration gradient drives transport by CTR.

Inspection of the available gene expression profiles of *R. irregularis* revealed that RiCTR2 is

up-regulated in *Medicago truncatula* colonized roots, suggesting that some Cu is mobilized from the internal stores in the IRM probably to provide Cu to Cu-binding proteins that might be required for fungal colonization (**Table 1**).

Copper-transporting P-type ATPases

Copper-transporting ATPases belong to the heavy metal P-type ATPase family (HMA), also known as P_{1B}-ATPases, which couples ATP hydrolysis to the efflux of positively charged metals from the cytoplasm. These proteins possess eight transmembrane domains, a large cytoplasmic loop, including ATP-binding and phosphorylation sites, and at least one conserved CPX motif (i.e., CPC) believed to be involved in metal cation translocation across the membrane. Four candidate genes putatively encoding Cu ATPases have been found in the *R. irregularis* genome, which represents an expansion compared with other fungi (**Tables 1** and **2**).

Table 2. Number and classification of the putative Cu transporters identified in the genome of *R. irregularis* and in the genomes of the reference fungi used in this study. CTR, Cu transporter. Modified from Tamayo et al. (2014).

	Basidiomycota							Ascomycota					Glomeromycota	Mucoromycotina
	Puccinia graminis	*Ustilago maydis*	*Laccaria bicolor*	*Coprinopsis cinerea*	*Cryptococcus neoformans*	*Piriformospora indica*	*Suillus luteus*	*Saccharomyces cerevisiae*	*Tuber melanosporum*	*Aspergillus niger*	*Neurospora crassa*	*Botrytis cinerea*	***Rhizophagus irregularis***	*Rhizopus oryzae*
CTR	2	1	2	4	2	3	3	3	2	4	4	3	3	3
P_{1B}-type ATPases	1	2	2	1	1	2	3	1	3	2	2	2	4	3

These proteins were clustered in two different groups in a phylogenetic tree (**Figure 3**). Three of them were grouped in a clade comprising the well characterized ortholog of *S. cerevisiae* CCC2, a protein that receives Cu from the Cu chaperone ATX1 via a direct protein–protein interaction, and pumps Cu into the late- or post-Golgi compartment to load Cu into a multicopper oxidase required for Fe uptake and, presumably, to other Cu-dependent proteins (Yuan et al., 1995). The other paralog, RiCRD1, groups in a different clade comprising various orthologs of CRD1, a plasma membrane Cu^{+} ATPase that plays a major role in Cu detoxification via Cu efflux in the opportunistic fungus *Candida albicans* (Weissman et al., 2000). Although it has been

suggested that functions of the fungal Cu-ATPases can be inferred from their positions in a phylogenetic tree (Saitoh et al., 2009), a detailed characterization of the *R. irregularis* paralogs is needed to understand their physiological functions.

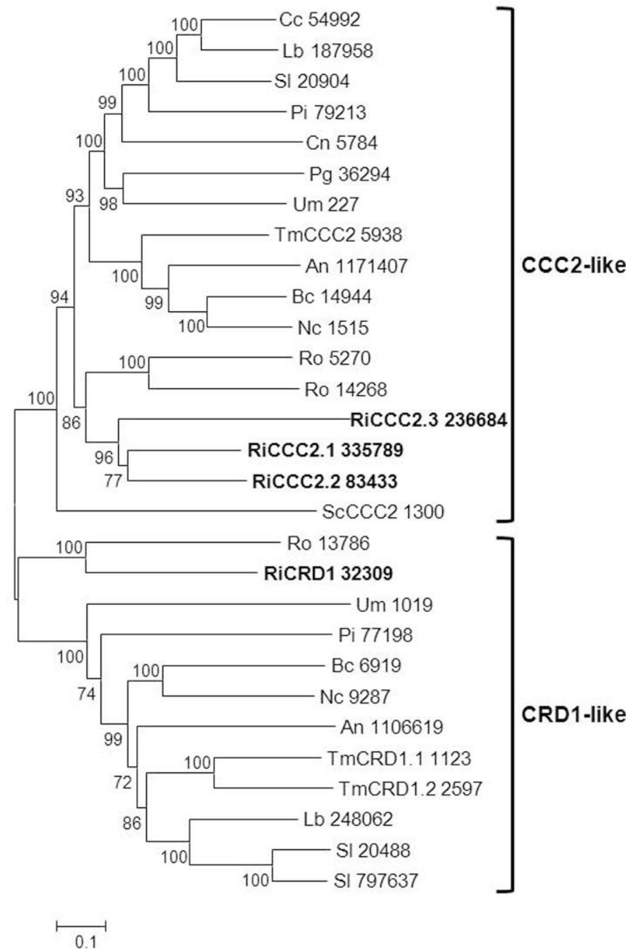


Figure 3. Phylogenetic relationships of the *Rhizophagus irregularis* copper-transporting P-type ATPases with homologous sequences from selected species representative of the major fungal phyla. The Neighbor-Joining tree was created with MEGA 6. Protein JGI identification numbers are indicated. *R. irregularis* genes are shown in bold. Organisms: An, *Aspergillus niger*; Bc, *Botrytis cinerea*; Cc, *Coprinopsis cinerea*; Cn, *Cryptococcus neoformans*; Lb, *Laccaria bicolor*; Nc, *Neurospora crassa*; Pi, *Piriformospora indica*; Pg, *Puccinia graminis*; Sc, *Saccharomyces cerevisiae*; Sl, *Suillus luteus*; Tb,*Tuber melanosporum*; Ri, *Rhizophagus irregularis*; Ro, *Rhizopus oryzae*; Um, *Ustilago maydis*. Bootstrap values above 70 and supporting a node used to define a cluster are indicated.

Analysis of the available expression profiles of *R. irregularis* revealed a 2.6-fold up-regulation of RiCCC2.1 gene expression in mycorrhizal roots relative to the expression levels in spores and ERM. No data are still available of the expression profiles of the other paralogs in the ERM. Up-regulation of RiCCC2.1 in the symbiotic stage, as it has been observed for the Cu transporter RiCTR2, suggests a role for these proteins to supply Cu to other enzymes required for fungal accommodation or functioning in the root tissues. In this respect, it has been shown that the

CTR2 ortholog of the plant pathogen *Colletotrichum gloeosporioides* (Barhoom et al., 2008) and the CCC2 orthologs of *Colletotrichum lindemuthianum* (Parisot et al., 2002) and *Botrytis cinerea* (Saitoh et al., 2010) are required for pathogenicity.

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CHAPTER II: The *Rhizophagus irregularis* genome encodes two CTR copper transporters that mediate Cu import into the cytosol and a CTR-like protein likely involved in copper tolerance

Adapted from:

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Introduction

The transition metal copper (Cu) is a micronutrient acting as a redox active cofactor of key enzymes involved in a wide array of biochemical processes essential for life, such as respiration, superoxide scavenging and iron mobilization (Linder, 1991; Festa et al., 2011). However, when in excess, it becomes toxic due to its ability to displace other metal ions in structural or catalytic protein motifs (Macomber and Imlay, 2009) and through the generation of hydroxyl radicals by Fenton-like reactions (Halliwell and Gutteridge, 1984). Due to the dual nature of Cu, organisms have developed sophisticated homeostatic networks to tightly regulate its intracellular levels, in which membrane transporters mediating Cu uptake and efflux and specific chaperones that handle and deliver Cu to its specific target enzymes play a key role (Puig and Thiele, 2002; Kim et al., 2008; Smith et al., 2017).

In eukaryotes, the major entrance of Cu into the cells occurs through members of the Cu transporter (CTR) family, small integral membrane proteins of variable length (from 150 to 500 amino acid residues) that have three transmembrane (TM) domains and the characteristic signature MetXXXMet-X_{12}-GlyXXXGly embedded within TM2 and TM3 (Dumay et al., 2006; De Feo et al., 2007). CTRs are present in the membranes as a homo-oligomer or hetero-oligomer complex, being the cooperation between the different subunits crucial for Cu transport (Puig et al., 2002; Beaudoin et al., 2011). The MetXXXMet motif is located in TM2 and together with a cluster of Met residues in the N terminal domain is involved in Cu sensing and uptake (Puig et al., 2002; Guo et al., 2004), while TM3 harbors the GlyXXXGly motif that is critical for protein folding and oligomerization (Aller et al., 2004). The carboxy terminal domain usually contains Cys and/or His motifs that bind and transfer Cu to cytosolic chaperones for its final targeted distribution (Dancis et al., 1994b; Xiao et al., 2004; Puig, 2014). Additionally, under Cu toxicity this domain allows protein inactivation through conformational structural changes (Wu et al., 2009). This family of transporters has been widely studied in *Saccharomyces cerevisiae*, which encodes three members (Ctr1, Ctr2 and Ctr3). Ctr1 and Ctr3 are functionally redundant plasma membrane transporters that mediate Cu acquisition from the environment (Dancis et al., 1994a; Peña et al., 2000), while Ctr2 is located in the vacuolar membrane and pumps vacuolar Cu stores to the cytosol (Portnoy et al., 2001; Rees et al., 2004). Apart from other yeasts (Bellemare et al., 2002; Marvin et al., 2003; Beaudoin et al., 2011), CTRs have been characterized in the basidiomycetes *Pleurotus ostreatus* (Penas et al., 2005), *Coprinopsis cinerea* (Nakagawa et al., 2010) and *Amanita strobiliformis* (Beneš et al., 2016), as well as in the filamentous ascomycetes *Podospora anserina* (Borghouts et al., 2002), *Colletotrichum gloeosporioides* (Barhoom et al., 2008) and *Neurospora crassa* (Korripally et al., 2010). However, very little is known about the mechanisms of Cu uptake in arbuscular mycorrhizal (AM) fungi, the most ancient and widespread fungal plant symbionts.

AM fungi are soil-borne microorganisms of the phylum Glomeromycotina that establish a mutualistic symbiosis with the majority of land plants. In this mutualistic relationship the fungal partner receives carbon compounds from the plant in exchange of low mobility mineral nutrients in soil, mainly phosphorus and some micronutrients, such as Zn and Cu (Smith and Read, 2008; Lanfranco et al., 2018). Besides improving plant mineral nutrition, AM fungi increase plant ability to overcome biotic and abiotic stress conditions, such as salinity, drought and metal toxicity (Ruiz-Lozano, 2003; Pozo et al., 2013; Ferrol et al., 2016). It is noteworthy the ability of AM fungi to increase plant fitness under deficient and excess Cu availability (Lehmann and Rillig, 2015; Ferrol et al., 2016). As revealed by isotopic labeling experiments, improvements in Cu nutrition by AM fungi are due to the capability of the extraradical mycelia (ERM) to absorb the micronutrient beyond the depletion zone that develops around the roots (Li et al., 1991; Lee and George, 2005). On the other hand, increased plant performance in Cu-polluted soils is mainly due to the ability of the fungus to act as a barrier for Cu entry into the plant tissues (Ferrol et al., 2016; Merlos et al., 2016). Despite the central role Cu transporters play in all organisms to cope with a range of Cu availability, from scarcity to excess, the mechanisms of Cu import in AM fungi have not been characterized yet. In a previous genome-wide analysis of metal transporters in the AM fungus *Rhizophagus irregularis*, we identified three genes putatively encoding Cu transporters of the CTR family that mediate Cu transport into the cytosol (Tamayo et al., 2014). With the aim to get further insights into the mechanisms of Cu homeostasis in AM fungi, in this work we carried out the first characterization of the *R. irregularis* CTR transporters. We show that the *R. irregularis* genome encodes two Cu transporters: RiCTR1 a plasma membrane transporter that mediates Cu uptake by the ERM and RiCTR2 a functional vacuolar transporter that is highly expressed during the *in planta* phase of the fungus. We also show that *RiCTR3* produces, as a consequence of an alternative splicing event, two transcripts, *RiCTR3A* and *RiCTR3B*, and that RiCTR3A might function as a Cu receptor involved in Cu tolerance.

Materials and Methods

Biological Materials and Growth Conditions

The AM fungal isolate used in this study was *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler DAOM 197198. The fungal inoculum used for the root organ cultures and for the seedlings was obtained in monoxenic cultures. Arbuscular mycorrhizal monoxenic cultures were established according to St-Arnaud et al. (1996), with some modifications. Briefly, Ri T-DNA transformed carrot (*Daucus carota* L. clone DC2) roots were cultured with *R. irregularis* in solid M medium (Chabot et al., 1992) in two-compartment Petri dishes. Cultures were started in one compartment by placing the fungal inoculum (ERM, spores

and mycorrhizal roots fragments) and some pieces of carrot roots. Plates were incubated in the dark at 24ºC for 6-8 weeks until the other compartment of the Petri dish was profusely colonized by the fungus and roots (root compartment). The older compartment was removed and filled with liquid M medium without sucrose (M-C medium) and the fungal mycelium was allowed to colonize this compartment (hyphal compartment) during the two subsequent weeks (Control plates).

For the Cu deficiency treatments, monoxenic cultures were established in media without Cu and started with roots and inoculum previously grown either in M media containing 0.5 µM $CuSO_4$ (moderate Cu deficiency treatment) or in M media without Cu (severe Cu deficiency treatment), and grown in the same conditions than the control plates but in media lacking Cu. ERM and mycorrhizal roots grown, respectively, in the hyphal and root compartment of each plate were collected, rapidly dried on filter paper, immediately frozen in liquid N and stored at -80ºC until used. An aliquot of the roots from each treatment was separated to estimate mycorrhizal colonization.

For the Cu toxicity and H_2O_2 treatments, the M-C medium of the hyphal compartment was removed and replaced with fresh liquid M-C medium (Control, 0.5 µM $CuSO_4$) or with M-C medium supplemented with 250 µM $CuSO_4$, 500 µM $CuSO_4$ or 1 mM H_2O_2 and incubated at 24ºC. The time of medium exchange was referred as time 0. Mycelia were collected 1, 2 and 7 days after Cu addition and 1 h after H_2O_2 supplementation.

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

R. irregularis ERM was also collected from mycorrhizal plants grown in the *in vivo* whole plant bidimensional experimental system described by Pepe et al. (2017) with some modifications. Briefly, chicory (*Cichorium intybus* L.) seeds were surface-sterilized and germinated for 10-15 days in sterilized sand. Seedlings were transplanted into 50 mL pots filled with sterilized sand and inoculated with spores, ERM and colonized roots obtained from monoxenic cultures. Pots were placed in sun-transparent bags (Sigma-Aldrich, B7026) and maintained during one month in a growth chamber at 24ºC/21ºC day/night and 16 h light photoperiod. The root system of each plant was cleaned, wrapped in a nylon net (41 µM mesh, Millipore NY4100010) and placed between two 13 cm membranes of mixed cellulose esters (0.45 µm pore diameter size, MF-Millipore HAWP14250) in 14 cm diameter Petri dishes having a hole on the edge to allow plant shoot growth and containing sterilized sand. Petri plates containing

plants were sealed with parafilm, wrapped with aluminum foil, placed into sun-transparent bags and maintained in a growth chamber. Plants were watered weekly with a 0.5X modified Hoagland nutrient solution containing 125µM KH_2PO_4 and 0.16 µM $CuSO_4$ (control treatment) or without Cu (Cu deficiency treatment). Each treatment consisted of five replicates. Petri dishes were opened 2 weeks after preparing the root sandwiches and ERM spreading from the nylon net onto the membranes was collected with tweezers, frozen in liquid N and stored at -80ºC until used. Roots wrapped in the nylon net were also frozen and stored at -80ºC. An aliquot of the roots was separated to estimate mycorrhizal colonization.

The *Saccharomyces cerevisiae* strains used in this study were MPY17 (*ctr1Δctr3Δ*), a double-mutant lacking the plasma membrane transporters Ctr1 and Ctr3 (Peña et al., 1998) and MPY17 *ctr2Δ* (*ctr1Δctr2Δctr3Δ*), a triple mutant lacking also the vacuolar transporter ScCtr2 (Rees et al., 2004) and WYT (*yap1Δ*) a strain lacking the transcription factor yap1 (Kuge and Jones, 1994). Detailed characteristics of yeast strains are listed in Supplementary **Table 1**. Yeast cells were maintained on YPD or minimal synthetic dextrose (SD) medium, supplemented with appropriate amino acids.

Table1. *Saccharomyces cerevisiae* strains used in this work.

Strain	Genotype	Reference
MPY17	*MATα ctr1::ura3::Kan^r ctr3::TRP1 his3 lys2-801 CUP1^R*	(Peña et al., 1998)
MPY17 *ctr2Δ*	*MATα ctr1::ura3::Kan^r ctr3::TRP1 ctr2::HIS3 lys2-801 CUP1^R*	(Rees et al., 2004)
WYT	*MATα his3 can1-100 ade2 leu2 trp1 ura3 yap1:: TRP1*	(Kuge and Jones, 1994)

Mycorrhizal Colonization

Histochemical quantificacion of mycorrhizal colonization was performed according to Trouvelot *et al.* 1986 using the Mycocalc program (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) in root samples previously cleared with 10% KOH and stained with 0.05% trypan blue (Phillips and Hayman, 1970). The abundance of the AM fungus was also assessed by determining the expression level of the *R. irregularis* elongation factor 1α (*RiEF1α*; GenBank Accession No. DQ282611), using as internal control the elongation factor 1α of the corresponding host plant (*Daucus carota* L. *DcEF1α*, GenBank Accession No. XM_017391845; *Cichorium intybus* L *CiEF1α,* GenBank Accession No. KP752079).

Nucleic Acids Extraction and cDNA Synthesis

R. irregularis genomic DNA was isolated from ERM developed in the hyphal compartment of control plates using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions.

Total RNA extraction from fungal ERM and mycorrhizal carrots roots developed in monoxenic cultures was performed using the Plant RNeasy Kit (Qiagen) following manufacturer's instructions. Total RNA from mycorrhizal chicory roots was isolated using the phenol/SDS method followed by LiCl precipitation (Kay et al., 1987). RNAs were DNase treated with the RNA-free DNase set (Qiagen) according to manufacturer's instructions and quantified using the Nanodrop 1000 Spectrophotometer (Thermo Scientific). cDNAs were synthetized from 1µg of total DNase-treated RNAs in a 20 µL reaction containing 200 U of SuperScript III Reverse Transcriptase (Invitrogen) and 2.5µM oligo $(dT)_{20}$ primer (Invitrogen), following the manufacturer's instructions.

RiCTRs Identification and Sequences Analyses

Candidate gene sequences putatively encoding RiCTRs were previously identified by Tamayo et al. (2014). Additional Blastp searches were performed in the filtered model datasets of the *R. irregularis* isolates DAOM197198 v2.0 and A1, A4, A5, B3 and C2 v1.0 (Chen et al., 2018) recently deposited at the JGI website (https://genome.jgi.doe.gov/portal/), using as a query the previously identified RiCTR candidates. These sequences were also used to identify CTR homologs via Blastp in the sequence datasets of other Glomeromycota species deposited on the JGI (*Gigaspora rosea* v1.0, *Rhizophagus cerebriforme* DAOM 227022 v1.0 and *Rhizophagus diaphanus* v1.0 (Morin et al., 2019)) and NCBI (*Diversispora epigaea* (Sun et al., 2019) and *Rhizophagus clarus* (Kobayashi et al., 2018)) websites.

Sequence analyses were performed using the DNAstar Lasergene software package (DNAstar), BLAST tool of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal Omega for sequence alignments (https://www.ebi.ac.uk/Tools/msa/clustalo/). Gene promoter sequences were screened for the presence of regulatory cis elements employing the tools included in the Promoter Database of *Saccharomyces cerevisiae* SCPD (http://rulai.cshl.edu/SCPD/). Specific Cu responsive elements (CuREs) and AP-1 sites were further screened through DNA pattern matching analyses in the fungal RSAT server (http://rsat-tagc.univ-mrs.fr/rsat/). Identity and Similarity percentages between proteins were calculated using Ident and Sim tool from Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/ident_sim.html). Conserved domains of proteins were identified using the Conserved Domain Database at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), predictions of putative transmembrane domains via the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), the SOSUI

engine v. 1.11 (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) and the TOPCONS web server (http://topcons.cbr.su.se/), Structural models of the RiCTRs were generated using MyDomains tool of Prosite (https://prosite.expasy.org/mydomains/). Phylogenetic analyses were performed via the Neighbor-Joining method implemented in the Molecular Evolutionary Genetics Analysis software v. 6. (MEGA), with 1000 bootstrap replicates, using Poisson model and pairwise deletion of gaps options for distance computation.

Gene Isolation

The cDNA sequences of the 5´and 3´ends of *RiCTR1-3* were confirmed and completed, when necessary, by RACE using the SMARTer® RACE 5'/3' kit (Clontech) according to the manufacturer's protocol. Genomic clones and full length cDNAs were obtained by PCR amplification of *R. irregularis* genomic DNA and cDNA, respectively, from ERM grown under control conditions in monoxenic cultures, using a set of primers flanking the complete open reading frames. PCR products were cloned into the pGEM-T Easy vector (Promega), following manufacturer's instructions. Plasmids were amplified by transformation of chemically *Escherichia coli* DH5α competent cells according to standard procedures and purified using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). All plasmids were checked by sequencing (ABI PRISM 3130*xl* Genetic Analyzer, Applied Biosystems, Carlsbard, USA).

Functional Complementation Analyses in Yeast

RiCTR open reading frames were sub-cloned between *Sma*I and *Xho*I sites (RiCTR1) or *Pst*I and *Sal*I sites (RiCTR2 and RiCTR3) of the yeast expression vector pDR196. For this purpose, the respective full-length cDNA sequences were flanked with the sequences recognized by the corresponding restriction enzymes by PCR. PCR products were cloned into the pGEM-T Easy vector (Promega), following manufacturer's instructions. The full-length cDNAs were then isolated from the pGEM-T Easy vector by digestion with the corresponding restriction enzymes and ligated into the digested pDR196 vector. All constructs were verified by sequencing. The *S. cerevisiae* strains *ctr1Δctr3Δ*, *ctr1Δctr2Δctr3Δ* and *yap-1Δ* were transformed with the corresponding pDR196-RiCTR constructs or with the empty vector using a lithium acetate-based method (Gietz and Schiestl, 2007). Transformants were selected in SD medium by autotrophy to uracil. For drop tests, transformants were grown to exponential phase in SD medium without uracil. Cells were harvested by centrifugation, washed twice and adjusted to a final OD_{600} of 1. Then, 5 µL of serial 1:10 dilutions were spotted on the corresponding selective medium. The transformed *ctr1Δctr3Δ* and *ctr1Δctr2Δctr3Δ* strains were spotted onto a non-fermentable carbon source ethanol-glycerol medium (YPEG: 1% yeast extract, 2% bacto-peptone, 2% ethanol, 3% glycerol, 1.5% bacto-agar) supplemented with 0, 10, or 20 µM $CuSO_4$. The transformed *yap-1Δ*

cells were spotted onto SD without uracil supplemented either with 1.5 mM $CuSO_4$ or 0.5 mM H_2O_2.

Protein Localization

Subcellular localization of RiCTR1-3 was assessed with N or C terminal fusions of these genes to the enhanced green fluorescent protein (eGFP) in the *S. cerevisiae* triple mutant *ctr1Δctr2Δctr3Δ* or in *yap-1Δ*. The coding sequences of *RiCTR1*, *RiCTR2* and *RiCTR3A* were cloned with or without their stop codon into pENTR/D-TOPO (Invitrogen) via TOPO reactions and then into the destination vectors pFGWDR196 or pGWFDR196 by using the Gateway LR Clonase recombination system (Invitrogen) for eGFP-tagging at the amino- or carboxy- terminus, respectively. The corresponding yeast mutants were transformed with the resulting pFGWDR/ pGWFDR196-RiCTR constructs or with the empty vector (negative control). Functionality of the GFP-tagged versions of RiCTRT1, RiCTR2 and RiCTR3A was tested in complementation assays, as previously described. For the protein localization assays, yeast cells were grown to exponential phase in liquid SD without uracil and visualized using a Leica TCS SP8 laser scanning microscope with a 63X oil N.A. 1.4 immersion objective. Emission fluorescence of GFP was excited at 488 nm and the emitted signal was collected between 500-540 nm. To reduce overexpression artifacts, yeast cells were treated just before visualization with the protein synthesis inhibitor cycloheximide (100 µM) for 45 minutes. Images were processed using ImageJ software.

Gene Expression Analyses

Gene expressions were analyzed by real-time quantitative RT-PCR using an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each 20 µl reaction contained 1 µl of a 1:10 dilution of the cDNA, 200 nM each primer and 10 µl iQ™ SYBR Green Supermix (Bio-Rad). The program consisted in an initial incubation at 95ºC for 3 min, followed by 38 cycles of 95ºC for 30 s, 58ºC for 30s and 72ºC for 30s, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat-dissociation protocol (from 58 to 95ºC) after the final PCR cycle. Since RNA extracted from mycorrhizal roots contains plant and fungal RNAs, specificity of the primers pairs was also analyzed by PCR amplification of carrot and chicory genomic DNA and cDNA from non-mycorrhizal carrot and chicory roots. Specificity of the RiCTR3A and RiCTR3B primer pairs was analyzed by PCR amplification of the *RiCTR3A* and *RiCTR3B* plasmid DNAs. RT-PCR determinations were performed in three independent biological samples with the threshold cycle (Ct) determined in duplicate in at least two independent PCR experiments. The relative abundance of the transcripts was calculated by using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized according to the expression of the *R. irregularis* elongation factor 1α (*RiEF1α*; GenBank Accession No. DQ282611).

Statistical Analyses

IBM SPSS Statistic software v.23 was used for the statistical analysis of the means and standard error determinations. Data were subjected to the Student´s t-test when two means were compared or by one-way ANOVA followed by a Fisher's LSD to find out differences among groups of means ($P < 0.05$). All the analyses are based on at least 3 biological replicates per each treatment ($n \geq 3$).

Results

Features of the* R. irregularis *CTR Proteins

The *R. irregularis CTR1*, *CTR2* and *CTR3* full-length cDNA sequences were obtained by RACE using gene-specific primers based on the sequences described by Tamayo et al. (2014) (GenBank Accession No. /JGI IDs: PKC06371/1491164 (*RiCTR1*), EXX67481/1726366 (*RiCTR2*) and PKC14368/ 495436 (*RiCTR3*)). Interestingly, two types of *CTR3* transcripts were identified in the *R. irregularis* ERM, *RiCTR3A* of 531 bp and *RiCTR3B* of 606 bp. Comparisons of the full-length cDNAs with the genomic sequences revealed the presence of three introns in *RiCTR1* and two in *RiCTR2* and *RiCTR3*, all of them flanked by the canonical splicing sequences GT and AG at the 5' and 3' ends, respectively (**Figure 1A**). Alignment of the *RiCTR3A* and *RiCTR3B* transcripts with the *RiCTR3* gene sequence indicates that both transcripts are alternatively spliced products of the same gene, as the *RiCTR3A* and *RiCTR3B* sequences are contained within the genomic sequence. *RiCTR3B*, the longest *RiCTR3* variant, contains the first intron after the *RiCTR3A* start codon generating a premature termination codon-containing mRNA. However, an additional start codon located within the second exon becomes available to produce a protein that contains the last 97 amino acids of RiCTR3A.

RiCTR1, *RiCTR2* and *RiCTR3A* encode proteins of 182, 170 and 176 amino acids, respectively, that have three TM domains with the MetXXXMet-X_{12}-GlyXXXGly signature embedded within TM2 and TM3, an intracellular loop connecting TM1 and TM2, the N terminus towards the extracellular space and the C terminus facing the cytosol (**Figures 1B,C**). RiCTR1 and RiCTR2 present a methionine motif, MetXXMet in RiCTR1 and MetXMet in RiCTR2, in the N terminal extracytosolic region 29 and 23 amino acids before TM1, respectively. This methionine motif, which is essential for CTR function, is absent in RiCTR3A. RiCTR2 harbors a cysteine/histidine motif in the carboxy-terminal region facing the cytoplasm, while RiCTR1 has a single histidine residue and RiCTR3A a cysteine and two histidine residues (**Figure 1C**). Despite the similar structure and sequence amino acid length of RiCTR1, RiCTR2 and RiCTR3A, similarity between their deduced amino acid sequences is lower than 53 %, displaying RiCTR1 and RiCTR3A the highest similarity (**Table 2**). *RiCTR3B* encodes a protein of 97 amino acids

that harbors the MetXXXMet-X_{12}-GlyXXXGly signature of CTR proteins, but has a single TM domain, the MetXXXMet is mislocalized in the N terminal domain and the GlyXXXGly motif is embedded in its only TM domain.

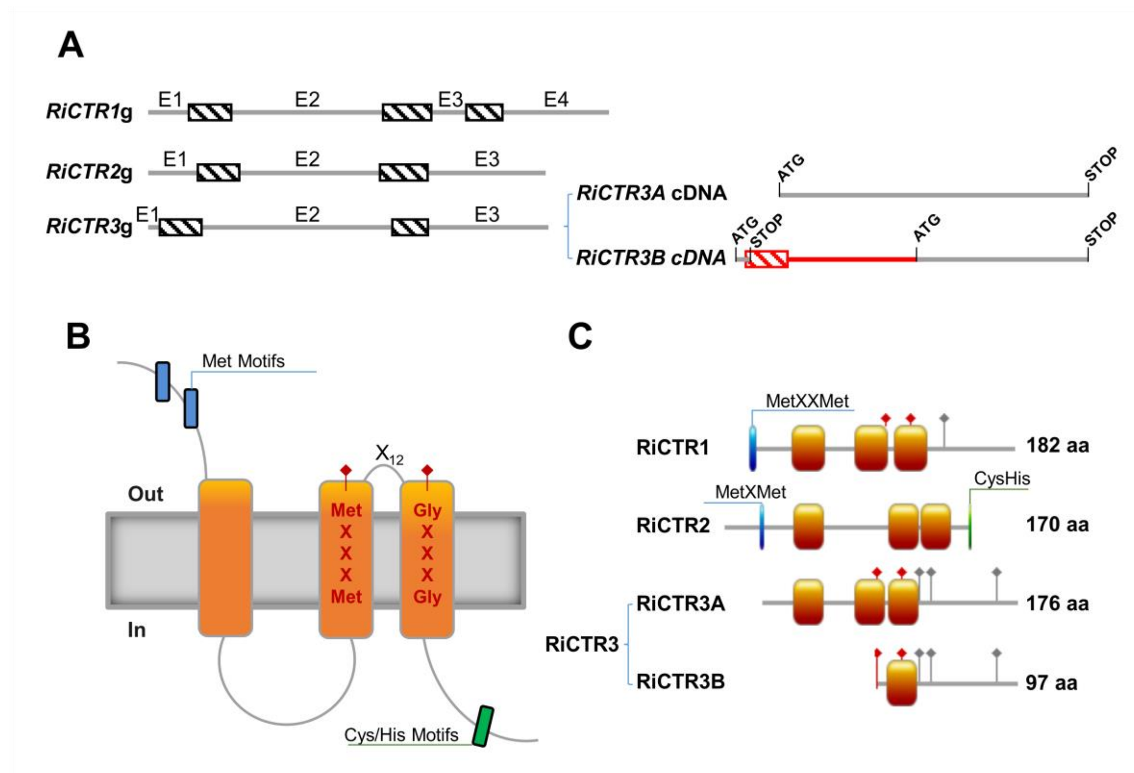

Figure 1. Structure of the *R. irregularis* CTR proteins. (**A**) Exon/intron organization of the *R. irregularis* CTR genes. Exons (E) and introns are represented by dashed boxes and gray lines, respectively. The two transcript variants of *RiCTR3* are illustrated at the right. The coding regions of the two spliced variants of *RiCTR3* are shown in grey and the non-coding in red. The start and stop codons are indicated. (**B**) Typical topological model of a CTR transporter. (**C**) Schematic representation of the structure of the *R. irregularis* CTR transporters. Orange boxes illustrate transmembrane domains, blue boxes Met motifs and green boxes Cys and His motifs. Red diamonds show the positions of the MetXXXMet and the GlyXXXGly motives of the CTRs master signature and gray diamonds the positions of the C-terminal His/Cys residues. Amino acid lengths are also indicated.

Table 2. Comparison among the predicted amino acid sequence of the *R. irregularis* CTR family members. Percentages of identity/similarity are indicated.

	RiCTR1	RiCTR2	RiCTR3A	RiCTR3B
RiCTR1	100/100	16/24	39/53	21/27
RiCTR2		100/100	11/20	4/9
RiCTR3A			100/100	55/55
RiCTR3B				100/100

A phylogenetic analysis of fungal CTR transporters revealed that RiCTR1 and RiCTR3 clustered with the *S. cerevisiae* plasma membrane Ctr1/Ctr3-like Cu transporters and RiCTR2 with the *S. cerevisiae* vacuolar Ctr2-like transporters. Within each clade, all Glomeromycota sequences were grouped together (**Figure 2**).

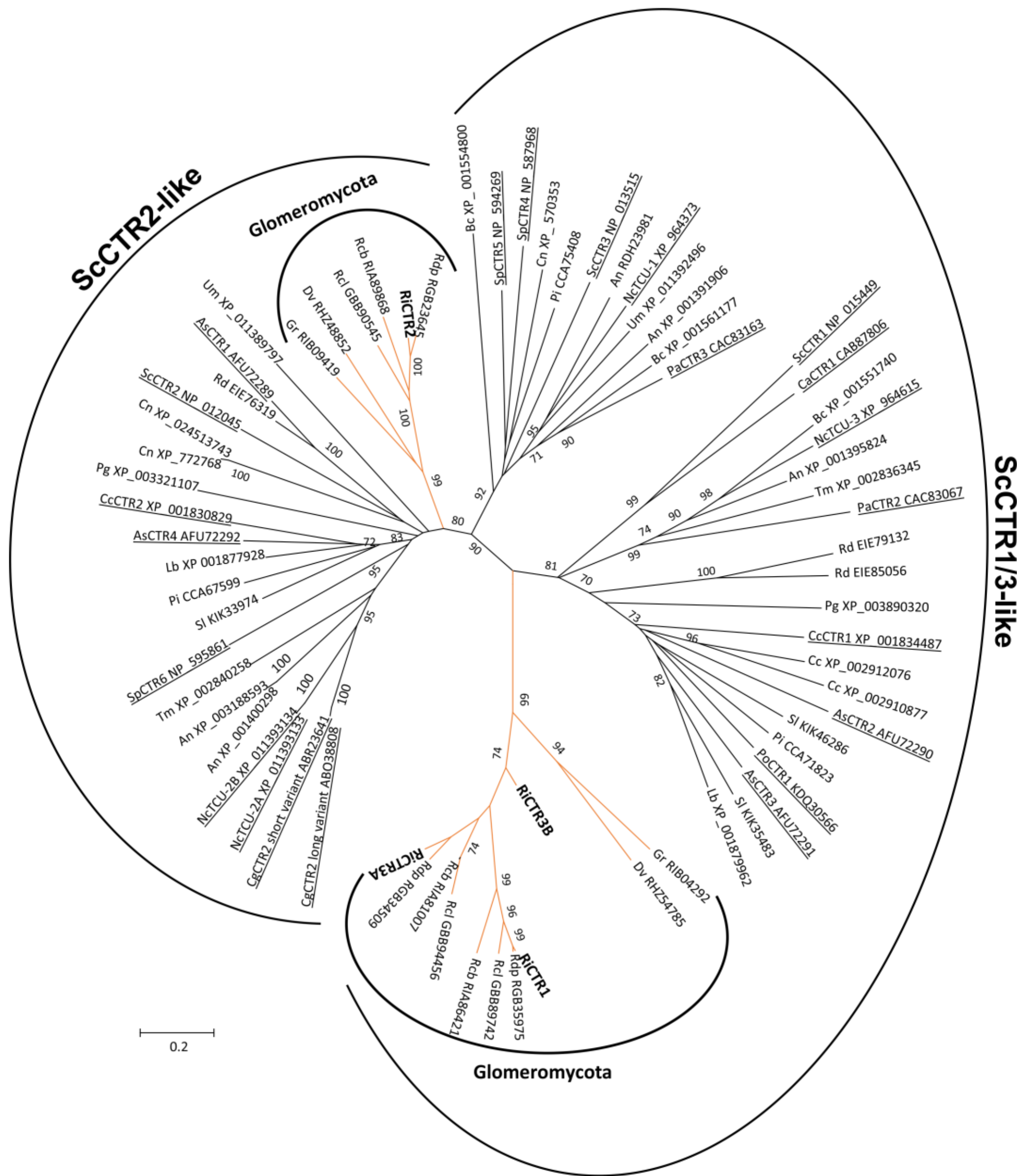

Figure 2. Phylogenetic relationships between fungal CTR proteins. The unrooted Neighbor-joining tree was generated using MEGA v. 6 with 1000 bootstrap replicates. *R.irregularis* CTR proteins are in bold. Functionally characterized CTRs are underlined. GenBank Accession numbers are provided. Organisms: As, *Amanita strobiliformis*; Bc, *Botrytis cinerea*; Ca, *Candida albicans*; Cc, *Coprinopsis cinerea*; Cg, *Colletotrichum gloeosporioides*; Cn, *Cryptococcus neoformans*; De, *Diversispora epigaea*; Gr, *Gigaspora rosea*; Lb, *Laccaria bicolor*; Nc, *Neurospora crassa*; Pa, *Podospora anserina*; Pi, *Piriformospora indica*;Pg, *Puccinia graminis*; Po, *Pleurotus ostreatus*; Sc, *Saccharomyces cerevisiae*; Sl, *Suillus luteus*; Sp, *Schizosaccharomyces pombe*; Tb, *Tuber melanosporum*; Rcb, *Rhizophagus cerebriforme*; Rcl, *Rhizophagus clarus*; Rdp, *Rhizophagus diaphanous*; Ri, *Rhizophagus irregularis*; Rd, *Rhizopus delemar*; Um, *Ustilago maydis*. Bootstrap values above 70 and supporting a node used to define a cluster are indicated.

In silico searches for putative regulatory elements in their promoter sequences resulted in the identification of several core elements identical to the Cu response cis-element (CuRE) GTAC present in the promoters of Cu-responsive genes (Jamison McDaniels et al., 1999; Kropat et al., 2005) and the consensus sequence of the AP-1 cis-acting element (TTATTAA/TTAGTAA) recognized as a conserved motif in the oxidative stress-responsive genes (Toone and Jones, 1999) (**Figure 3**). Interestingly, the 5´-flanking region of *RiCTR3* is especially rich in AP-1 motifs and contains the preferred DNA binding site (TTACTAA) of the transcription factor YAP1 (Toone and Jones, 1999), which is essential for the oxidative stress response in *S. cerevisiae* (Kuge and Jones, 1994).

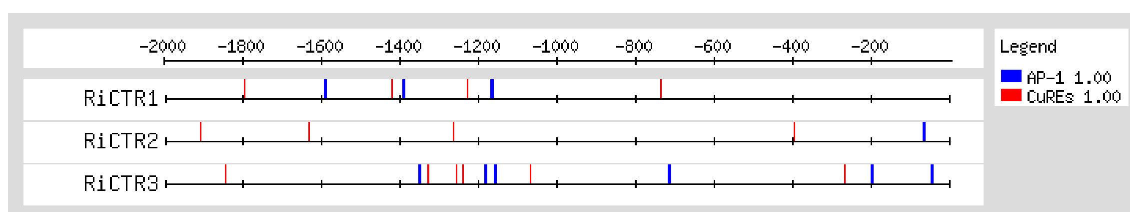

Figure 3. Putative cis-regulatory elements in the promoter region of *Rhizophagus irregularis CTRs* genes. Cis-regulatory elements were screened in the *Saccharomyces cerevisiae* Promoter Database SCPD (http://rulai.cshl.edu/SCPD/) and specific Cu responsive elements (CuREs) and AP-1 sites oxidative stress related DNA binding sites where further screened through DNA pattern matching analyses in the fungal RSAT server without allowing any nucleotide substitution (http://rsat-tagc.univ-mrs.fr/rsat/).

RiCTR1 ***and*** **RiCTR2** ***Encode Functional Cu Transporters***

Since AM fungi cannot be genetically manipulated, functionality of the RiCTRs was assessed in yeast by testing their ability to revert the inability of the double (*ctr1Δctr3Δ*) and triple (*ctr1Δctr2Δctr3Δ*) *S. cerevisiae* CTR mutants, which lack the plasma membrane Ctr1 and Ctr3 Cu transporters and the plasma membrane Ctr1/Ctr3 and the vacuolar Ctr2 transporters, to grow on a non-fermentable carbon source at low Cu concentrations. This growth defect is due to the inability of the cytochrome c oxidase to obtain its Cu cofactor, resulting in a defective mitochondrial respiratory chain (Dancis et al., 1994a; Glerum et al., 1996; Rees et al., 2004). To perform the yeast complementation assays, the full-length cDNA coding sequences of *RiCTR1*, *RiCTR2*, *RiCTR3A* or *RiCTR3B* were expressed under the control of the yeast PMA1 promoter in both yeast CTR mutants and plated on ethanol-glycerol (YPEG) medium supplemented with different Cu concentrations. The empty vector-expressing cells were unable to grow on YPEG medium containing < 20 µM Cu (**Figure 4A**). Expression of *RiCTR1* restored the inability of the *ctr1Δctr3Δ* and *ctr1Δctr2Δctr3Δ* yeast strains to grow on YPEG medium supplemented with 10 µM Cu, indicating that RiCTR1 is a functional homolog of the yeast plasma membrane Cu transporters Ctr1/Ctr3. RiCTR2 complemented the inability of the *ctr1Δctr2Δctr3Δ* mutant strain to grow on YPEG medium supplemented with 10 µM Cu but not of the double mutant lacking

the two plasma membrane transporters, which suggests that RiCTR2 is a functional homologue of the *S. cerevisiae* vacuolar transporter Ctr2. However, none of the *RiCTR3* variants rescued the phenotype of either the double or the triple CTR mutants (**Figure 4A**), which was expected since their encoded proteins did not contain the required features for CTR function.

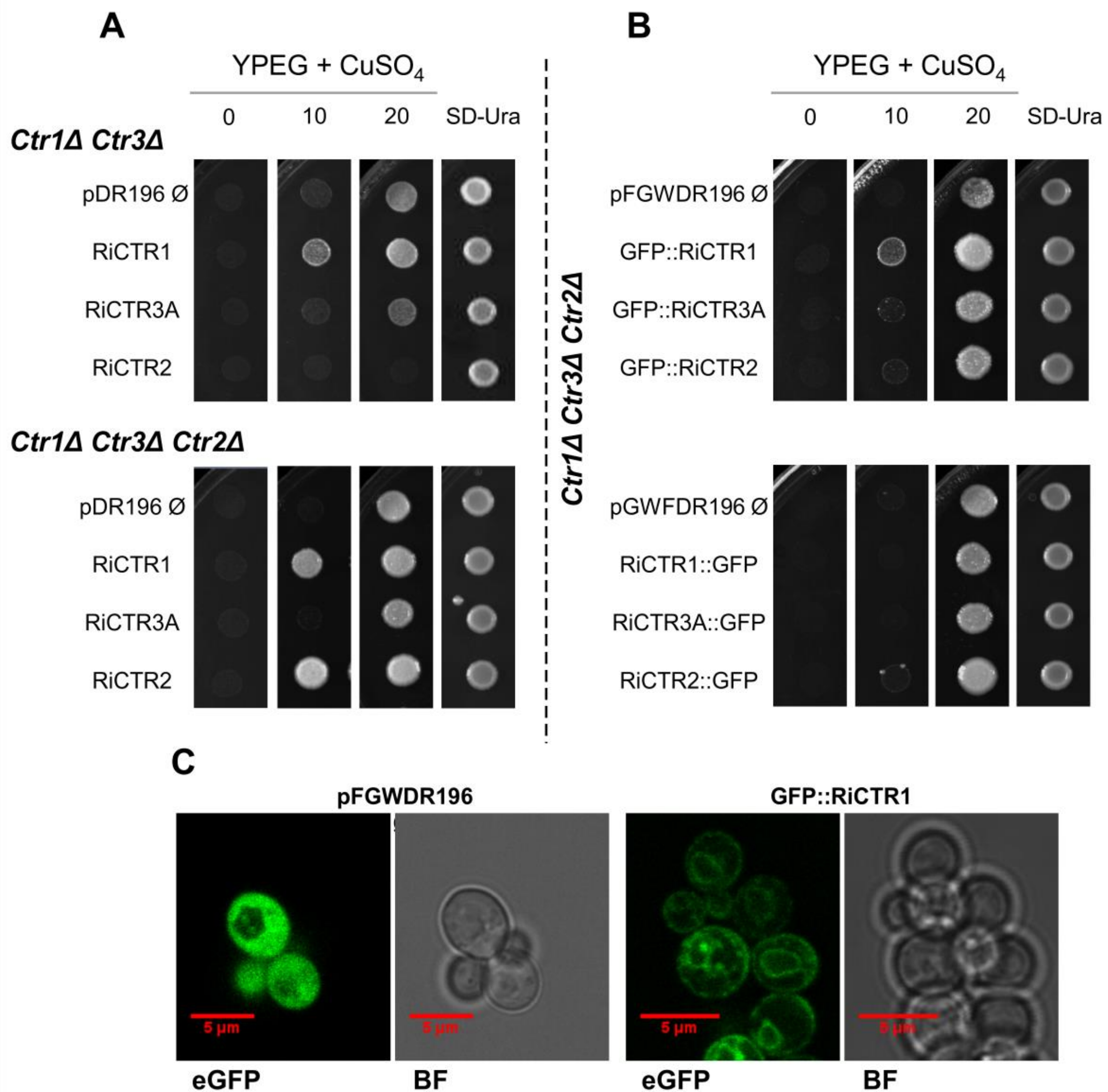

Figure 4. Analysis of the Cu transport activity and subcellular localization of the *R. irregularis* CTRs in yeast. (**A**) *ctr1Δctr3Δ* and *ctr1Δctr2Δctr3Δ* yeast cells transformed with the empty vector or expressing *RiCTR1*, *RiCTR2*, *RiCTR3A or RiCTR3B* were plated on YPEG media supplemented with Cu (0, 10 or 20 µM $CuSO_4$) or on SD medium without uracil. *ctr1Δctr3Δ* and *ctr1Δctr2Δctr3Δ* plated cells were incubated at 30 °C for 4 and 7 days, respectively. RiCTR3A and RiCTR3B had the same effect, only the result with RiCTR3A is illustrated. (**B**) *ctr1Δctr2Δctr3Δ* yeast cells expressing GFP (empty vector), N-terminal (upper panel) or C-terminal (lower panel) GFP-tagged versions of *RiCTR1*, *RiCTR2* and *RiCTR3A* were plated on YPEG media supplemented with Cu (0, 10 or 20 µM $CuSO_4$) or on SD medium without uracil. Plates were incubated at 30 °C for 7 days. (**C**) *ctr1Δctr2Δctr3Δ* cells expressing GFP (the empty vector pFGWDR196) and GFP::RiCTR1 were visualized with a confocal microscope. eGFP: GFP fluorescence, BF: Bright Field.

RiCTR ***Genes are Differentially Expressed in the IRM and ERM***

Subcellular localization of RiCTR1 and RiCTR2 was assessed in the heterologous system by expressing N- and C-terminal GFP-tagged versions of these proteins in the *ctr1Δctr2Δctr3Δ* strain and visualizing the fusion proteins with a confocal fluorescence microscope. *S. cerevisiae* cells transformed with the empty vector and expressing GFP under the control of the PMA1 promoter were used as a negative control; and functionality of the RiCTR1-2 fusion proteins was assessed before their visualization (**Figure 4B**). The control cells expressing the soluble GFP showed a general cytosolic fluorescence (**Figure 4C**). The RiCTR1-GFP, RiCTR2-GFP and GFP-RiCTR2 fusion proteins were unable to revert the mutant phenotype of the ctr*1Δctr2Δctr3Δ* strain and were expressed within the perinuclear endoplasmic reticulum region (data not shown), indicating that the fusion proteins failed to exit the endoplasmic reticulum. As expected from the complementation assays, the *GFP-RiCTR1*-expressing cells showed a clear fluorescent signal at the cell periphery indicative of a plasma membrane localization. GFP-RiCTR1 was also localized within the perinucelar endoplasmic reticulum membrane, a phenomenon commonly found in yeast membrane protein overexpression assays (**Figure 4C**).

To gain information about the expression of *RiCTR1-3* during symbiosis and about their relative abundance in the intraradical mycelia (IRM) and ERM, their expression levels were determined by real time quantitative RT-PCR (RT-qPCR) in ERM grown in liquid monoxenic cultures and in the *in vivo* sandwich system, and in the IRM developed in carrot roots grown *in vitro* for two weeks on a densely colonized hyphal compartment and devoid of ERM and in mycorrhizal chicory roots collected from the *in vivo* sandwich system. Mycorrhizal colonization levels of the carrot and chicory roots were 10 and 78 %, respectively. As a reference for fungal activity, we measured transcript levels of the *R. irregularis* high-affinity monosaccharide transporter *RiMST2,* which is highly expressed in the IRM during AM symbiosis (Helber et al., 2011). In both experimental systems, *RiCTR1* was the isoform more highly expressed in the ERM and the expression levels of *RiCTR2* were higher in the IRM than in the ERM. Expression levels of the two spliced-variants of *RiCTR3* were very low in both fungal structures and more highly expressed in the ERM (**Figure 5**).

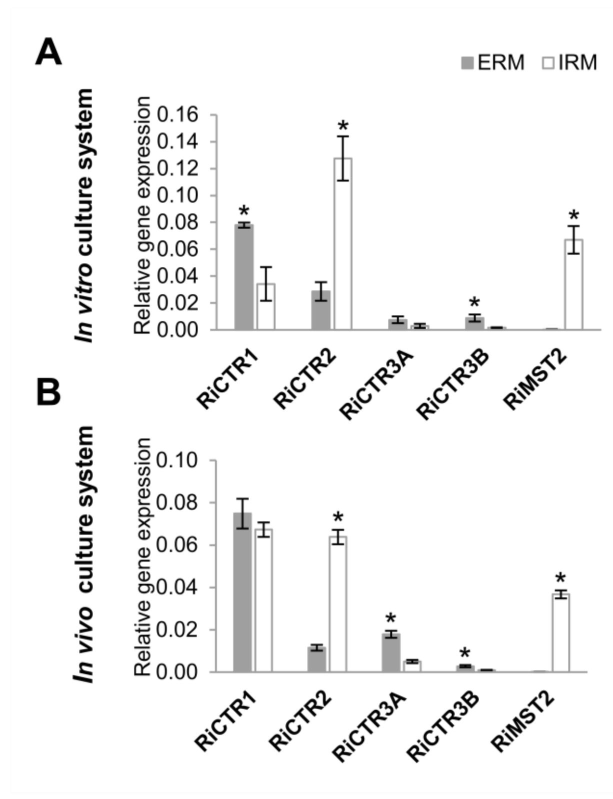

Figure 5. *RiCTR*s expression levels in the *R. irregularis* ERM and IRM. (**A**) *R. irregularis* ERM and mycorrhizal carrot roots (IRM) were grown in compartmented monoxenic cultures (*in vitro* culture system). (**B**) *R. irregularis* ERM and mycorrhizal chicory roots (IRM) were grown in the whole plant bidimensional experimental system (*in vivo* culture system). Relative gene expression levels were calculated by the $2^{-\Delta CT}$ method using *RiEF1α* as a normalizer. Bars represent standard error. Asterisks show statistically significant differences ($P < 0.05$) between ERM and IRM.

Cu Deficiency Inhibits AM Colonization and Regulates RiCTR *Expression in the IRM*

To further understand the role of RiCTR1-3 in the intraradical phase of the fungus, their expression levels were analyzed in mycorrhizal carrot roots grown *in vitro* in monoxenic cultures and in mycorrhizal chicory roots grown *in vivo* in the sandwich system under Cu-optimal and -deficient conditions. Interestingly, irrespective of the culture method, development of the roots under Cu-deficient conditions decreased mycorrhizal intensity and arbuscule frequency **(Figure 6**). These results were confirmed by determining the transcript levels of the *R. irregularis* elongation factor *RiEF1α* by qRT-PCR (**Figure 6**).

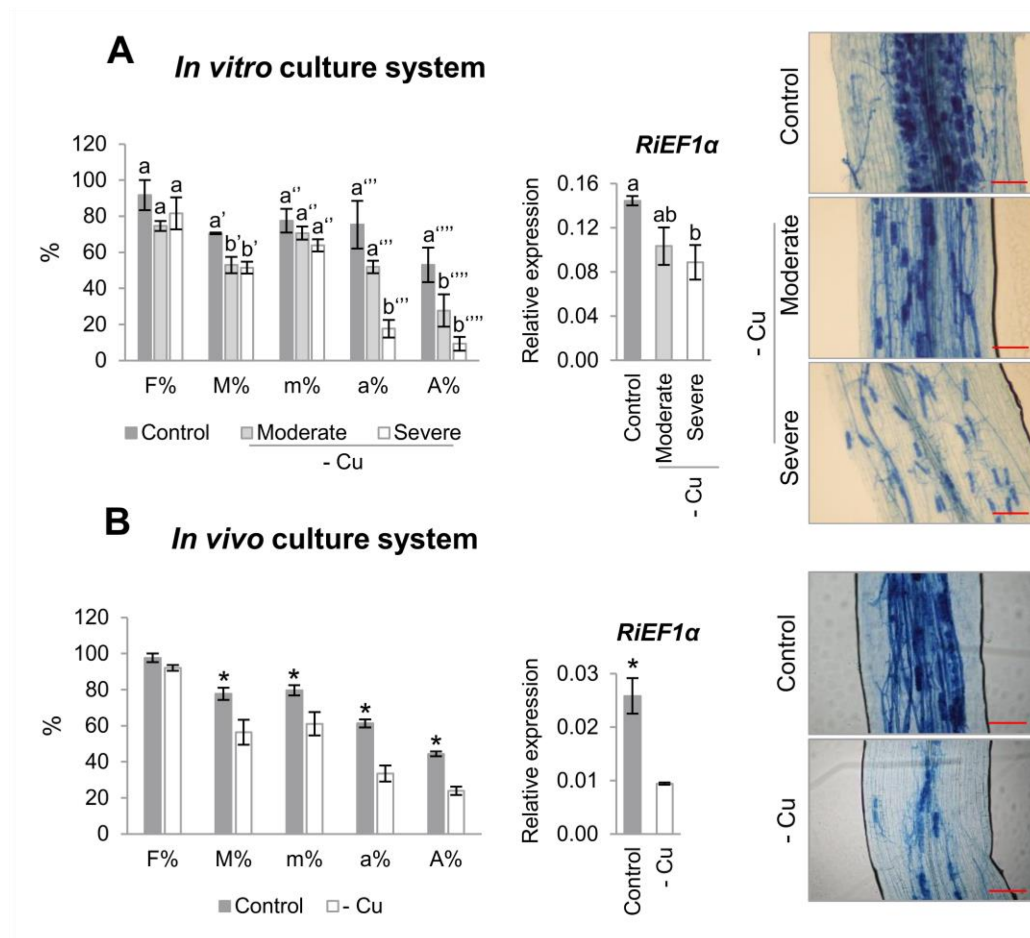

Figure 6. Effect of Cu deficiency on mycorrhizal colonization. (**A**) Mycorrhizal colonization of carrot roots grown in monoxenic cultures in M media (control, 0.5 µM Cu) or in M media lacking Cu in plates started either with roots and inoculum previously grown in M media containing 0.5µM $CuSO_4$ (moderate Cu deficiency) or without Cu (severe Cu deficiency). (**B**) Mycorrhizal colonization of chicory roots grown in the whole plant bidimensional experimental system fertilized with half-strength Hoagland solution (control, 0.16 µM Cu) or with a modified nutrient solution without Cu. Colonization rates were determined by using the Trouvelot method after histochemical staining and by determining the expression level of the *R. irregularis* elongation factor 1α (*RiEF1α*). The relative expression of Ri*EF1α* was calculated using the $2^{-\Delta CT}$ method with *EF1α* of the corresponding host plant as internal control. Bars represent standard error. Different letters indicate significant differences ($P < 0.05$) between treatments and asterisks statistically significant differences ($P < 0.05$) in comparison with the control. Scale bar of the left panels:100 µm.

Cu deficiency increased *RiCTR1* expression in the IRM developed in the carrot root organ cultures and in the chicory roots grown in the sandwich system (**Figure 7A**). However, *RiCTR*2 expression was only up-regulated in the IRM of the carrot roots grown under the severe Cu deficiency treatment and of the chicory roots fed with a nutrient solution without Cu (**Figure 7B**). None of the RiCTR3 splicing variants were detected in the mycorrhizal roots grown under Cu-limiting conditions, probably because their low expression levels in the IRM and the decrease in mycorrhizal colonization.

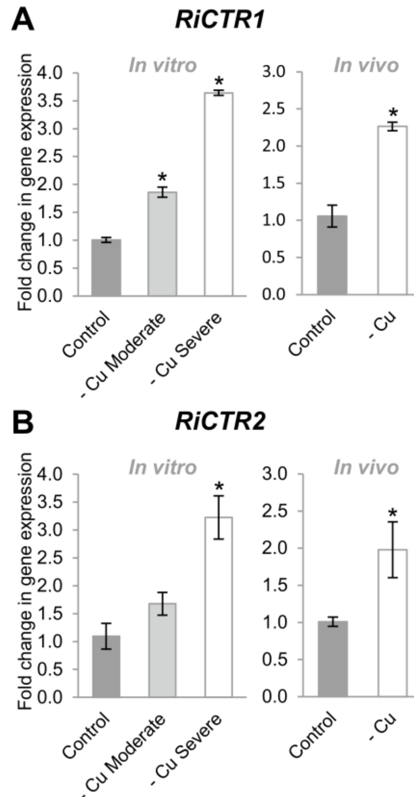

Figure 7. Effect of Cu deficiency on *RiCTR1* and *RiCTR2* IRM expression. (**A**) *RiCTR1* and (**B**) *RiCTR2* expression in mycorrhizal carrot roots developed in monoxenic cultures in M media (control, 0.5 µM Cu) or in M media lacking Cu in plates started either with roots and inoculum previously grown in M media containing 0.5µM $CuSO_4$ (moderate Cu deficiency) or without Cu (severe Cu deficiency) (left panel) and in mycorrhizal chicory roots grown in the whole plant bidimensional experimental system and fertilized with half-strength Hoagland solution (control, 0.16 µM Cu) or with a modified nutrient solution without Cu (right panel). Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using *RiEF1α* as a normalizer. Bars represent standard error. Asterisks show statistically significant differences ($P < 0.05$) in comparison to the corresponding control value.

RiCTRs *Expression in the ERM is Regulated by Cu Availability*

To get further insights into the role of the *R. irregularis* CTR family members on fungal Cu homeostasis, their gene expression patterns in extraradical mycelia (ERM) grown monoxenically under Cu deficient and toxic conditions. Given that development of the ERM was seriously inhibited when the hyphal compartment of the split Petri dishes was supplied with high Cu levels (data not shown), the Cu toxicity treatments were applied by exposing the ERM grown in M media to 250 µM $CuSO_4$ for 1, 2 or 7 days or to 500 µM $CuSO_4$ for 1 and 2 days.

RiCTR1 expression was up-regulated by Cu deficiency and down-regulated by Cu toxicity. A 2-fold induction was observed in the ERM grown both under moderate and severe Cu limiting conditions (**Figure 8A**). In contrast, *RiCTR2* transcript levels were significantly increased (2-fold) only in the ERM grown under the severe Cu-limiting treatment. *RiCTR2*

expression was not affected by any of the toxic Cu conditions considered in our study (**Figure 8B**).

The expression levels of *RiCTR3A* and *RiCTR3B* were similar in the control untreated ERM. Interestingly, *RiCTR3A* expression was highly up-regulated in ERM subjected to the Cu toxicity treatments and down-regulated in the ERM grown under Cu limiting conditions. *RiCTR3A* induction by Cu toxicity seemed to be transient, reaching a maximum expression level (>20 fold induction) in the ERM exposed to 500 μM $CuSO_4$ for one day (**Figure 8C**). This expression pattern was unexpected for a gene encoding a protein that transports Cu into the cytosol and suggests a role for RiCTR3A in Cu tolerance. *RiCTR3B* expression was just slightly up-regulated in the ERM grown for 7 d at 250 μM Cu and for 1 d at 500 μM Cu (**Figure 8D**). Differential regulation of the two *RiCTR3* splicing variants by Cu leads to higher transcript levels of *RiCTR3A* than of *RiCTR3B* under Cu toxic levels and to higher transcript levels of *RiCTR3B* under the severe Cu deficient treatment.

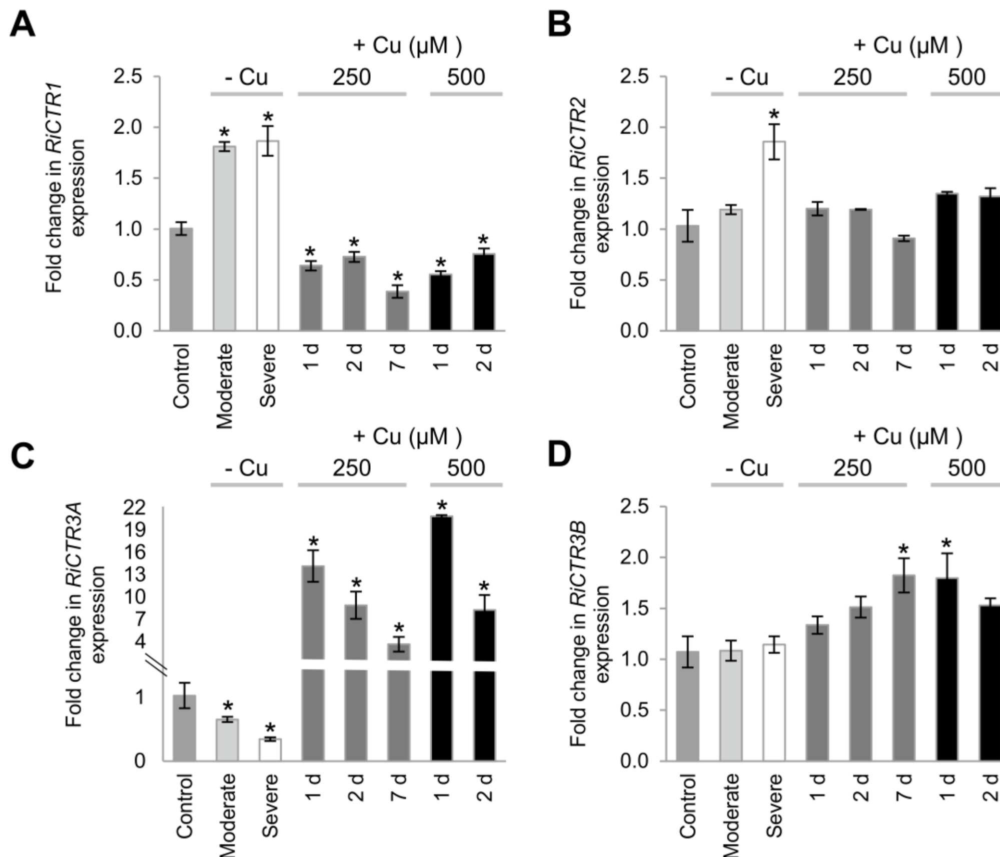

Figure 8. Regulation of *RiCTRs* expression by Cu availability. *R. irregularis* ERM was grown in monoxenic cultures in M media containing 0.5μM $CuSO_4$ (control) or in M media lacking Cu in plates started with roots and inoculum previously grown either in M media containing 0.5μM $CuSO_4$ (moderate Cu deficiency) or in M media without Cu (severe Cu deficiency). For the Cu toxicity treatments, the ERM grown in optimal M media was exposed for 1, 2 or 7 days to 250 500 μM $CuSO_4$ or for 1 and 2 days to 500 μM $CuSO_4$. (**A**) *RiCTR1*, (**B**) *RiCTR2*, (**C**) *RiCTR3A* and (**D**) *RiCTR3B* gene expression. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using *RiEF1α* as a normalizer. Bars represent standard error. Asterisks show statistically significant differences ($P < 0.05$) in comparison to the corresponding control value.

RiCTRs *Expression is Regulated by Oxidative Stress*

Taking into account that several oxidative-stress response elements were identified in the promoter sequences of the *R. irregularis CTR* genes and that toxic Cu levels induce an oxidative stress to the ERM (Benabdellah et al., 2009), in an attempt to further understand the role of the *R. irregularis* CTRs, their gene expression patterns were analyzed in the ERM exposed to H_2O_2. As a marker of the oxidative stress treatment, the expression of the *R. irregularis* Cu,Zn superoxide dismutase gene *RiSOD1* (González-Guerrero et al., 2010) was determined. Exposure of the ERM to 1 mM H_2O_2 for 1 h up-regulated *RiCTR1, RiCTR2, RiCTR3B* and *RiSOD1* expression (**Figures 9 A,B,D,E**). However, *RiCTR3A* expression was not significantly affected by H_2O_2, which indicates that its activation by Cu was independent of the Cu-induced oxidative stress (**Figure 9C**).

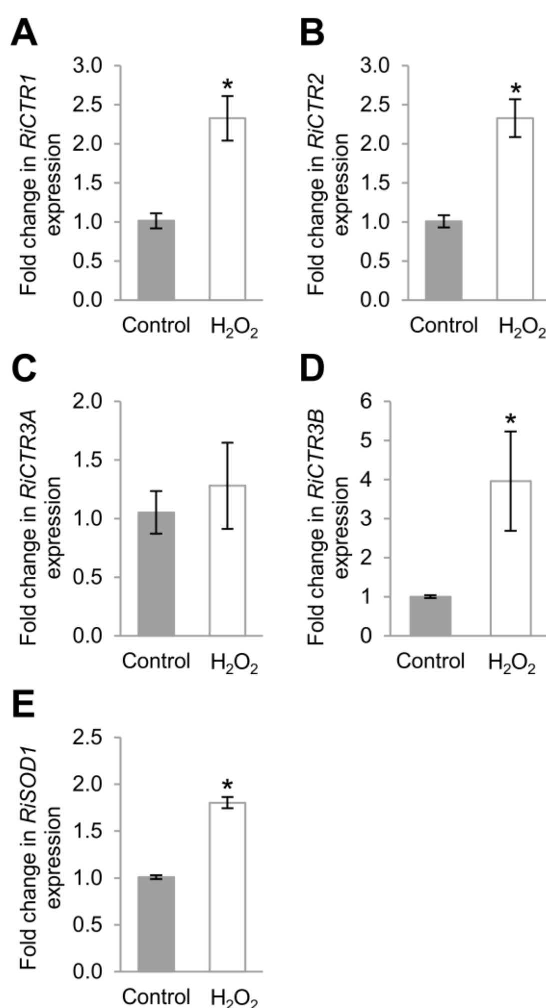

Figure 9. Regulation of *RiCTRs* expression by oxidative stress. *R. irregularis* ERM grown in monoxenic cultures in M-C medium was exposed or not (Control) for 1 h to 1mM H_2O_2. (**A**) *RiCTR1*, (**B**) *RiCTR2*, (**C**) *RiCTR3A* (**D**) *RiCTR3B* and (**E**) *RiSOD1* gene expression. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using *RiEF1α* as a normalizer. Bars represent standard error. Asterisks show statistically significant differences ($P < 0.05$) in comparison to the control value.

RiCTR3A Enhances Metal Tolerance of the* yap1Δ *Yeast Strain

As a step forward to understand RiCTR3A and RiCTR3B function and taking into account that their transcript levels were regulated by Cu toxicity or H_2O_2, we assessed their capability to rescue metal and H_2O_2 sensitivity of the *yap1Δ S. cerevisiae* strain lacking the transcriptional regulator Yap1 that mediates cell´s response to oxidants and metals. Neither the empty vector-transformed cells nor the *RiCTR3B*-expressing cells were able to grow on SD media supplemented with Cu or H_2O_2 (**Figure 10A**). However, RiCTR3A rescued the growth defect of the mutant yeast in media supplemented with 1.5 mM $CuSO_4$ but not the inability of the *yap1Δ* cells to grow in the presence of 0.5 mM H_2O_2 (**Figure 10A**). These data indicate that RiCTR3A plays, at least in the heterologous system, a role in Cu tolerance

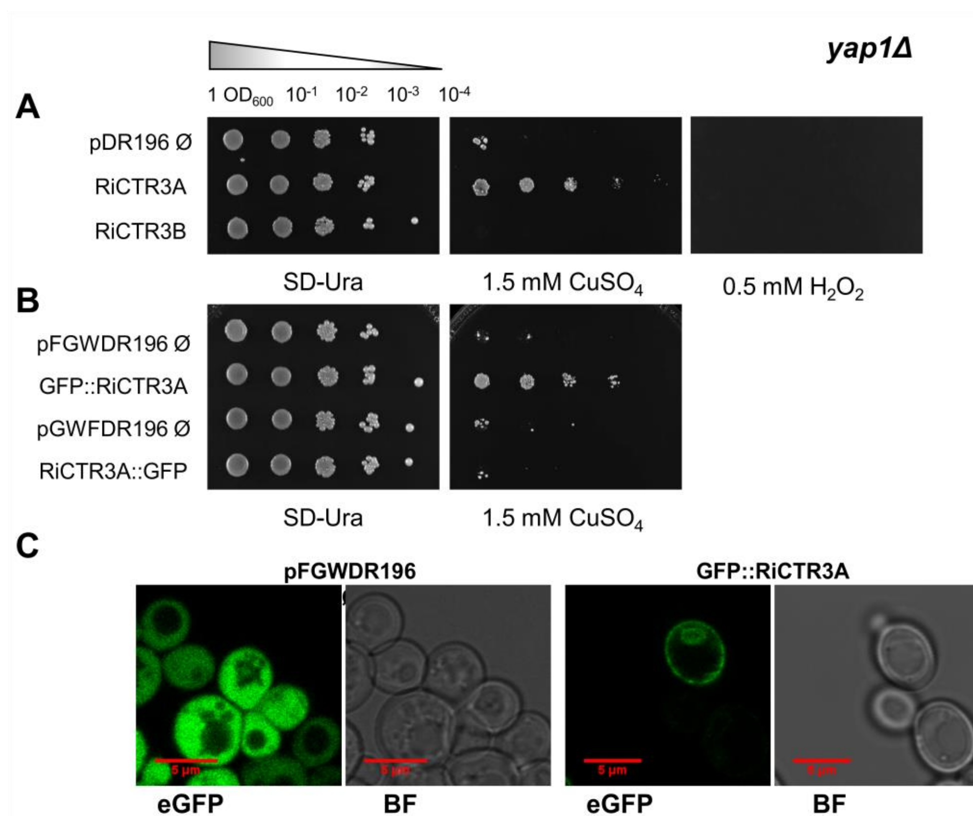

Figure 10. Functional analysis of RiCTR3A and RiCTR3B in the *Δ-yap1 S. cerevisiae* mutant. (**A**) *Δ-yap1* cells transformed with the empty vector or expressing RiCTR3A or RiCTR3B were plated on SD media supplemented or not with 1.5 mM $CuSO_4$ or with 0.5 mM H_2O_2. (**B**) *Δ-yap1* cells transformed with the corresponding empty vector expressing GFP or N-terminal or C-terminal GFP-tagged versions of RiCTR3A were plated on SD media supplemented or not with 1.5 mM $CuSO_4$. Plates were incubated 4 days at 30ºC. (**C**) *Δ-yap1* cells expressing GFP (the empty vector pFGWDR196) and GFP::RiCTR3A were visualized with a confocal microscope. eGFP: GFP fluorescence; BF: Bright Field.

To determine RiCTR3A subcellular location in *yap1Δ*, N- and C-terminal GFP-tagged versions of RiCTR3A were expressed in the mutant yeast cells. However, only GFP-RiCTR3A-GFP remained functional (**Figure 10B**). This fusion protein was localized to the yeast plasma

membrane. Additionally, as usually occurs when transport proteins are overexpressed in yeast, a perinuclear fluorescence pattern indicative of endoplasmic reticulum localization was observed (**Figure 10C**).

Discussion

The ability of AM fungi to acquire Cu from the soil and to transfer it to their host plants has been shown in several physiological studies. Whereas much progress has been made in understanding the mechanisms of phosphorus and nitrogen transport in the AM symbiosis, very little is known about the mechanisms of Cu acquisition by AM fungi. Here, we characterize for the first time the Cu transporters of the CTR family in an AM fungus. Our data strongly suggest that *R. irregularis* acquires Cu (I) from the soil through the activity of RiCTR1, a plasma membrane Cu transporter that is highly expressed in the ERM, and that RiCTR2 and RiCTR3A play a role in Cu homeostasis in *R. irregularis*.

A previous genome-wide analysis of Cu transporters in *R. irregularis* revealed the presence of three candidate gene sequences, *RiCTR1*, *RiCTR2* and *RiCTR3*, encoding transporters belonging to the CTR family (Tamayo et al., 2014). Interestingly, our RACE approach identified two *RiCTR3* transcripts, which result from an alternative splicing event through the retention of the first intron in its coding sequence, the most common alternative splicing type described in fungi (Grutzmann et al., 2014; Gonzalez-Hilarion et al., 2016). Alternative splicing is a common mechanism used to produce multiple proteins from a single gene, thereby increasing the proteome size of an organism (Black, 2003; Benabdellah et al., 2007; Kornblihtt et al., 2013; Mockenhaupt and Makeyev, 2015). In addition, it can influence gene expression through its impact on different stages of mRNA metabolism including transcription, polyadenylation, nuclear mRNA export, translation efficiency and the rate of mRNA decay (Le Hir et al., 2003; Jacob and Smith, 2017). Although functionality of alternative splicing is poorly understood in fungi, it seems that usually leads to non-functional isoforms, providing an additional mechanism to regulate the overall expression of a gene (Goebels et al., 2013; Grutzmann et al., 2014; Gonzalez-Hilarion et al., 2016; Jin et al., 2017; Sieber et al., 2018). However, the extent and biological significance of this process in AM fungi is currently unknown.

CTR proteins contain three TM regions, with a characteristic MetXXXMet motif located in the second TM domain that is absolutely necessary for Cu transport, and an amino-terminal region rich in methionine motifs (De Feo et al., 2007). Although most of these methionine motifs are dispensable for Cu transport, a Met/Cys-X-Met motif near the first TM domain is essential for function (Puig et al., 2002). Our *in silico* analyses revealed that out of the four identified RiCTR open reading frames, only RiCTR1 and RiCTR2 present all the structural features of CTR

proteins. In fact, these two proteins were the only *R. irregularis* CTRs displaying Cu transport activity in the heterologous system. The finding that RiCTR1 reverts the mutant phenotype of the *ctr1Δctr3Δ* strain lacking the high affinity plasma membrane Cu transporters *Ctr1* and *Ctr3* indicates that RiCTR1 encodes a high affinity plasma membrane Cu transporter that transports Cu (I). Localization of RiCTR1 in the yeast plasma membrane and *RCTR1* expression patterns in the ERM in response to external Cu, that is up-regulation by Cu deficiency and down-regulation by Cu toxicity, supports this hypothesis. Although RiCTR2 subcellular localization could not be demonstrated in the heterologous system, it seems to be the functional ortholog of the *S. cerevisiae* vacuolar Cu transporter Ctr2, as it complemented the growth defect of the triple CTR mutant yeast *ctr1Δctr2Δctr3Δ* lacking both the vacuolar and plasma membrane transporters but not of the double mutant lacking only the plasma membrane transporters. These data strongly suggest a role for RiCTR2 in mobilization of vacuolar Cu stores, which is supported by up-regulation of *RiCTR2* expression when the ERM was grown under the severe Cu deficient conditions.

Interestingly, *RiCTR1* and *RiCTR2* transcript levels raised in the ERM in response to H_2O_2. A potential explanation could be that under these conditions RiCTR1 and RiCTR2 are needed to increase Cu availability for Cu/Zn-superoxide dismutase, one of the cofactors needed for its reactive oxygen species scavenging activity. In fact, yeast cells lacking CTR transporters show oxidative stress sensitive phenotypes linked with an insufficient delivery of Cu, either from the external environment or from the vacuolar reserves, to the Cu/Zn superoxide dismutase (Dancis et al., 1994a; Knight et al., 1996). These data suggest, therefore, a role for Cu in oxidative stress protection in *R. irregularis*.

As reported for the *R. irregularis* genes *RiPT* (Fiorilli et al., 2013) *RiAMT1-3* (Pérez-Tienda et al., 2011; Calabrese et al., 2016) and *RiFTR1* (Tamayo et al., 2018) encoding, respectively, plasma membrane phosphate, ammonium and iron transporters, *RiCTR1* and *RiCTR2* mRNAs were detected in the IRM. Expression of *RiCTR1* in the IRM suggests, as it has been proposed for the other fungal transporters, that there might exist a competition between the plant and the fungus for the Cu present in the apoplast of the symbiotic interface (Balestrini et al., 2007; Kiers et al., 2011). It is tempting to hypothesize that during its *in planta* phase, the fungus needs to take up Cu from the interfacial apoplast to meet its Cu demands for growth and activity. This hypothesis is supported by the observed increase of the *RiCTR1* transcript levels in the IRM when the symbiosis was developed under Cu-limiting conditions. The high expression levels of *RiCTR2* in the IRM together with its up-regulation when the symbiosis was developed under Cu-deficient conditions suggest that the fungus needs to mobilize its vacuolar Cu reserves to support its growth and metabolism. Overall, these data indicate that the fungus has a high Cu demand for growth and activity within the roots, which is supported by the observation that root colonization and arbuscule development are inhibited when the symbiosis was developed under Cu-deficient

conditions. The requirement of Cu for AM fungal colonization it is not surprising given that this transition metal is an essential micronutrient that acts as cofactor of key enzymes involved in a wide array of biochemical processes essential for growth (Pena et al., 1999; Festa et al., 2011).

Unlike RiCTR1 and RiCTR2, none of the *RiCTR3* gene products seem to have a role in Cu transport. RiCTR3A presents the typical topology of CTR proteins but lacks the conserved Met/Cys-X-Met motif near the first TM domain that is strictly required for Cu transport; and RiCTR3B has a single TM domain. As expected, neither RiCTR3A nor RiCTR3B restored the respiratory defect of the CTR *S. cerevisiae* mutant yeasts. Furthermore, their gene expression patterns in response to external Cu presented the opposite trend of a protein that mediates Cu transport into the cytosol, as both were transiently up-regulated when the ERM was exposed to high Cu levels. The strong up-regulation of *RiCTR3A* expression in the Cu-treated ERM together with the capability of its gene product to revert metal sensitivity of the *Δyap-1* yeast cells suggest that RiCTR3A is involved in metal tolerance in the ERM. Given that RiCTR3A was unable to complement oxidative stress sensitivity of the *Δyap-1* mutant and that a functional GFP-RiCTR3A fusion protein was localized to the *Δyap-1* plasma membrane, it is temping to hypothesize that RiCTR3A might function as a Cu sensor that activates downstream signal transduction pathways involved in Cu tolerance. Nutrient sensing in fungi is mediated by different classes of plasma membrane proteins that activate downstream signaling pathways, such as non-transporting receptors, transceptors and G-proteins-coupled receptors (Van Dijck et al., 2017). Non-transporting receptors are structural homologs to nutrient transporters that have lost their transport capacity while gaining a receptor function (Conrad et al., 2014). It is believed that these transporter-like proteins are used as sensors for the nutrient they likely once transported previously in evolution. This is the case of the *S. cerevisiae* glucose receptors Snf3 and Rgt2, structural homologs glucose transporters that sense availability of external glucose but cannot transport glucose (Ozcan et al., 1998), and of the nitrogen receptor SSy1, a member of the amino acid permease family that does not transport amino acids but senses them at the plasma membrane (Klasson et al., 1999). Despite failure of RiCTR3A to complement the mutant phenotype of the yeast CTR mutants could be an artifact of the heterologous system, the absence in its N-terminal end of the Met/Cys-X-Met motif that is strictly required for CTR function supports the hypothesis that RiCTR3A does not have Cu transport activity. Although micronutrient receptors have been not reported yet, the yeast iron transporter Ftr1 and the zinc transporter Ztr1 have recently been identified as the first micronutrient transceptors, since they present both transport and receptor functions (Schothorst et al., 2017). RiCTR3A might be the first described micronutrient receptor. However, further studies are required to confirm this hypothesis.

Unfortunately, we could not assign a role to *RiCTR3B*, the intron-retaining transcript of *RiCTR3*. Alternative splicing variants of CTR genes have been previously described in other

fungi, such as *C. gloeosporioides* (Barhoom et al., 2008) and *N. crassa* (Korripally et al., 2010). However, in contrast with what happens with the protein encoded by *RiCTR3B*, the predicted proteins of the two spliced variants of the *C. gloeosporioides CTR2* gene and of the *N. crassa* TCU-2 present all the characteristic features of CTR proteins and their gene products are fully functional in the yeast Ctr triple mutant. Since intron retention in *RiCTR3B* produces a frame shift that generates a premature termination codon, it is possible that the alternative *RiCTR3* protein RiCTR3B is not produced. If that were the case, as it has been described for other fungi (González-Hilarion et al., 2016), intron retention might be a post-transcriptional mechanism to regulate *RiCTR3* gene expression. A systemic genome-wide comparative analysis of alternative splicing in 23 fungal species has revealed that most of the alternative splicing-affected genes encode proteins that mediate the stress response (Grutzmann et al., 2014). Interestingly, RiCTR3A seems to be involved in the ERM response to Cu toxicity. The finding that both *RiCTR3* splicing variants were differentially expressed during Cu and oxidative stress agrees with previous observations in several human pathogenic fungi that the expression of a certain isoform is not exclusive to a certain condition and that the ratio between expressed isoforms changes (Sieber et al., 2018). These authors suggested that alternative splicing is important in fungi for adaptation and stress tolerance via the generation of suitable splice variants. The higher expression levels of the *RiCTR3A,* the transcript lacking the first intron, under Cu toxicity suggests that alternative splicing may be a mechanism to control the activation of the RiCTR3A protein during Cu stress. The finding that *RiCTR3B* expression increased in the H_2O_2-exposed ERM suggests that it might play a role in oxidative stress tolerance. However, further studies are needed to determine whether *RiCTR3B* encodes a functional protein and the significance of the alternative splicing of *RiCTR3*.

Conclusion

Here, we show for the first time that the AM fungus *R. irregularis* expresses two genes encoding Cu transporters of the CTR family, *RiCTR1* and *RiCTR2,* and two alternative spliced variants of a third gene, *RiCTR3*. *RiCTR3A*, the shortest spliced variant of *RiCTR3*, encodes a protein that is likely involved in Cu tolerance while *RiCTR3B* might contribute to oxidative stress protection. Our data also show for the first time the requirement of Cu for AM fungal colonization.

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CHAPTER III: RiCRD, a *Rhizophagus irregularis* Cu efflux heavy metal ATPase with a dual key role on Cu detoxification and symbiotic Cu nutrition

Introduction

Copper (Cu) homeostasis is tightly controlled in all organisms due to its duality as an essential micronutrient, but toxic when in excess. Cu is actively used as a cofactor by cytochrome c oxidases, superoxide dismutase and multicopper oxidases, among other enzymes, which are involved in important processes such as respiration, reactive oxygen species (ROS) removal and Fe nutrition, respectively (Linder, 1991). The key role of Cu in metabolic processes is associated with its ability to fluctuate between the oxidized (Cu^{+2}) and reduced (Cu^{+1}) state, resulting in the acceptance and donation of single electrons in cellular redox reactions. However, these redox properties also make this metal toxic when present at high concentrations. Cu excess can damage DNA, proteins and lipids through the generation of ROS by Fenton like reactions and can displace other metals cofactors (Halliwell and Gutteridge, 1984; Macomber and Imlay, 2009). Although Cu is a trace element, owing to anthropogenic activities, Cu toxicity has become an agricultural and environmental problem in recent years. High Cu concentrations are toxic to soil inhabitants, but some soil microorganisms have developed adaptative mechanisms that allow them to survive and grow in environments with high Cu concentrations (Bååth, 1989; Ferrol et al., 2009)

Arbuscular mycorrhizal () fungi, obligate biotrophs of higher plants, constitute one of the most prominent groups of soil AM microorganisms (Barea et al., 2005). AM fungi belong to phylum Glomeromycota and establish a widespread mutualistic symbiosis with most land plants (Brundrett and Tedersoo, 2018; Wijayawardene et al., 2018). The fungus biotrophically colonizes the root cortex and develops specialized structures called arbuscules to facilitate nutrient exchange between symbionts (Luginbuehl and Oldroyd, 2017). Simultaneously, the fungus develops an extensive network of extraradical hyphae that can absorb nutrients beyond the depletion zone that develops around the roots, providing a new pathway, the mycorrhizal pathway, for the uptake of low mobility macronutrients (P, N, K) and micronutrients (Cu, Zn) in soil (Ferrol et al., 2018; Lanfranco et al., 2018; Wipf et al., 2019). In return for delivering mineral nutrients, the obligate biotrophic AM fungus receives up to 20 % of the photosynthetically fixed carbon from the plant (Roth and Paszkowski, 2017).

In soils with low Cu levels, the contribution of the mycorrhizal pathway to plant Cu nutrition can be up to 75% (Li et al., 1991; Lee and George, 2005).We have recently shown that Cu uptake by the ERM is mediated by a plasma membrane Cu transporter of the CTR family of Cu transporters whose expression in the ERM increases under Cu deficiency but decreases under Cu toxicity. However, it is currently unknown how Cu is transferred to the plant in the arbuscule-colonized cortical cells.

On the other hand, under conditions of supraoptimal levels of Cu, AM fungi are able to alleviate metal toxicity in the plant. Different mechanisms have been proposed to explain the

protective effect of the AM symbiosis in heavy metal stress (Ferrol et al., 2016). One of the mechanisms of the attenuated effect of Cu is reduction of the effective metal concentration that is available to the plant through metal immobilization in the intraradical and extraradical structures (González-Guerrero et al., 2008; Cornejo et al., 2013). This is possible thanks to the existence in the fungus of a complex regulatory system that controls Cu homeostasis and avoids Cu stress in the cytosol. These mechanisms include binding of the metal to the cell wall, reduction of metal uptake, intracellular buffering through the activity of intracellular chelators, such as metallothioneins and glutathione, and Cu compartmentalization in the vacuoles or in the spores (Ferrol et al., 2016). However, a Cu efflux strategy has not been described yet.

Export of metal ions, such as Cu, Zn and Cd, usually takes place through P_{IB}- type ATPases, proteins that couple ATP hydrolysis to the transport of a heavy metal across different cellular membranes in a multistep process, which includes the specific recognition of the metal (Palmgren and Nissen, 2011). The genome of the model fungus *Rhizophagus irregularis* has four candidate genes putatively encoding four putative P_{1B}-type ATPases (Tamayo et al., 2014). *RiCCC2.1-3* are orthologs of the *Saccharomyces cerevisaie CCC2* Cu-ATPase that transports Cu to Cu containing proteins in the trans-Golgi region (Yuan et al., 1995), while *RiCRD1* is the ortholog of the P_{1B}-ATPase *CaCRD1* of the pathogenic yeast *Candida albicans* that export Cu excess out of the cell providing Cu resistance (Riggle and Kumamoto, 2000; Weissman et al., 2000). With the aim of determining whether RiCRD1 might play a role in Cu release to the apoplast of the symbiotic interface and/or in metal tolerance, the expression patterns of *RiCRD1* were analyzed in the *R. irregularis* IRM and ERM developed in the presence of different Cu levels.

Materials and Methods

Biological materials and growth conditions

Rhizophagus irregularis (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler DAOM 197198 monoxenic cultures were established with Ri T-DNA transformed carrot (*Daucus carota* L. clone DC2) roots in two-compartment Petri dishes filled with solid M medium (Chabot et al., 1992), according to (St-Arnaud et al., 1996) with some modifications. Briefly, cultures were started in one compartment of the Petri dish by placing some non-mycorrhizal carrots roots fragments and the fungal inoculum containing ERM fragments of mycorrhizal roots and spores. Plates were incubated in the dark at 24ºC for 6-8 weeks until the other compartment was densely colonized by the fungus and roots (root compartment). The oldest compartment was removed and filled with liquid M medium without sucrose (M-C medium) and the fungal

mycelium was allowed to colonize this compartment (hyphal compartment) during the two subsequent weeks (Control plates).

For the Cu deficiency treatments, monoxenic cultures were established in media without Cu and started with roots and inoculum previously grown in M media without Cu. ERM and mycorrhizal roots grown, respectively, in the hyphal and root compartment of each plate were collected, rapidly dried on filter paper, immediately frozen in liquid N and stored at -80ºC until used. An aliquot of the roots from each treatment was separated to estimate mycorrhizal colonization.

For the Cu and Cd toxicity treatments, the M-C medium of the hyphal compartment was removed and replaced with fresh liquid M-C medium (Control, 0.5 µM $CuSO_4$) or with M-C medium supplemented with 250 µM $CuSO_4$, 500 µM $CuSO_4$ or 45 µM $CdSO_4$ and incubated at 24ºC. The time of medium exchange was referred as time 0. Mycelia were collected 1, 2 and 7 days after Cu addition and 1, 3, 6, 12, 24 and 48 hours after Cd supplementation.

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

R. irregularis ERM was also collected from mycorrhizal plants grown in the *in vivo* whole plant bidimensional experimental system described by Pepe et al. (2017) with some modifications. Briefly, chicory (*Cichorium intybus* L.) seeds were surface-sterilized and germinated for 10-15 days in sterilized sand. Seedlings were transplanted into 50 mL pots filled with sterilized sand and inoculated with spores, ERM and colonized roots obtained from monoxenic cultures. Pots were placed in sun-transparent bags (Sigma-Aldrich, B7026) and maintained during one month in a growth chamber at 24ºC/21ºC day/night and 16 h light photoperiod. The root system of each plant was cleaned, wrapped in a nylon net (41 µM mesh, Millipore NY4100010) and placed between two 13 cm membranes of mixed cellulose esters (0.45 µm pore diameter size, MF-Millipore HAWP14250) in 14 cm diameter Petri dishes having a hole on the edge to allow plant shoot growth and containing sterilized sand. Petri plates containing plants were sealed with parafilm, wrapped with aluminum foil, placed into sun-transparent bags and maintained in a growth chamber. Plants were watered weekly with a 0.5X modified Hoagland nutrient solution containing 125µM KH_2PO_4 and 0.16 µM $CuSO_4$ (control treatment) or without Cu (Cu deficiency treatment). Each treatment consisted of five replicates. Petri dishes were opened 2 weeks after preparing the root sandwiches and ERM spreading from the nylon net onto the membranes was collected with tweezers, frozen in liquid N and stored at -80ºC until used.

Roots wrapped in the nylon net were also frozen and stored at -80ºC. An aliquot of the roots was separated to estimate mycorrhizal colonization.

Mycorrhizal colonization

Mycorrhizal colonization was assessed after trypan blue staining (Phillips and Hayman, 1970) according to Trouvelot *et al.* (1986) method. The abundance of AM fungus in the roots was also determined molecularly by determining the expression levels of the *R. irregularis* elongation factor 1α (*RiEF1α*; GenBank Accession No. DQ282611) using as internal control the elongation factor 1α of the corresponding host plant (*Daucus carota* L. *DcEF1α*, GenBank Accession No. XM_017391845; *Cichorium intybus* L *CiEF1α,* GenBank Accession No. KP752079).

RNA isolation and cDNA synthesis

The Plant RNeasy Kit (Qiagen) was used to extract total RNA from the ERM and mycorrhizal carrots roots developed in monoxenic following the manufacturer's instructions. Total RNA from mycorrhizal chicory roots was extracted using the phenol/SDS method followed by LiCl precipitation as described by Kay et al. (1987). The isolated RNAs were DNase treated with the RNA-free DNase set (Qiagen) according to the manufacturer's instructions and quantified with the Nanodrop 1000 Spectrophotometer (Thermo Scientific). 1 µg of each RNA was used for the cDNA synthesis in a 20 µL final volume reaction containing 200 U of SuperScript III Reverse Transcriptase (Invitrogen) and 2.5µM oligo $(dT)_{20}$ primer (Invitrogen), following the manufacturer's instructions.

Real-Time quantitative RT-PCR

Gene expression patterns were analyzed in an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad) using iQ™ SYBR Green Supermix (Bio-Rad). The program consisted in an initial incubation at 95ºC for 3 min, followed by 38 cycles of 95ºC for 30 s, 58ºC for 30s and 72ºC for 30s, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat-dissociation protocol (from 58 to 95ºC) after the final PCR cycle. Since RNA extracted from mycorrhizal roots contains plant and fungal RNAs, specificity of the primers pairs was also analyzed by PCR amplification of carrot and chicory genomic DNA and cDNA from non-mycorrhizal carrot and chicory roots. The relative abundance of the transcripts was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized with the *R. irregularis* elongation factor 1α (*RiEF1α*; GenBank Accession No. DQ282611). All determinations were performed in at least three biological samples with the threshold cycle (Ct) determined in duplicate in at least two independent PCRs.

In situ ***hybridization of*** **RiCRD** ***transcripts in mycorrhizal roots***

200 bp sense and antisense probes of *RiCRD* and 18S RNA were generated by two nested PCR reactions using gene-specific primers containing a 5´overhang to allow their fusion to the T7 RNA polymerase promoter sequence. The first PCR was carried out on cDNA from ERM grown under control conditions in monoxenic cultures with the primer pairs RiCRD-T7-Pup and RiCRD-Pdown or RiCRD-Pup and RiCRD-T7-Pdown. The second PCR was performed using 1 µL of a 1/100 dilution of the amplicon and the primer pairs E-T7 and RiCRD-Pdown or RiCRD-Pup and E-T7. Both amplifications were performed with GoTaq®G2 DNA polymerase (Promega) in a final volume reaction of 25 µL, using the following protocol: 94ºC for 3 min, 40 cycles of [94ºC for 30s, 56 ºC for 30s and 72 ºC for 30s]. Amplification products were purified by ethanol precipitation and used to obtain digoxigenin-UTP-labelled RNA probes using the MAXIscript® T7 Transcription Kit following manufacturer´s instructions (Invitrogen). 18S sense and antisense ribosome probes were used as a positive control.

Hybridization and detection of the probes were performed on 8 µm-thick sections of 8 weeks-old mycorrhizal tomato roots (*Lycopersicon esculentum* cv. Moneymaker). Tomato plants were inoculated with a substrate based *R.irregularis* inoculum (10%) containing mycorrhizal root fragments, spores and mycelia.

Root fragments of 3 mm were fixed overnight at 4ºC in 4% (w/v) paraformaldehyde, 0.1% Triton X-100 in phosphate-buffered saline (PBS) followed by to 2 successive 5 min of vacuum infiltration. Roots were washed with PBS 3 times for 30 min, dehydrated through incubations in graded ethanol-butanol series and immobilized in paraffin (ParaplastPlus, Leica BioSystems). Longitudinal and cross-sections of 8 µm-thickness were obtained using a microtome Leica RM2255, mounted on silanized slides (Euromedex) and completely dried. Sections were deparaffinized with three 10 min Safesolv washes (Labonord), rehydrated (ethanol 100% 3 times, 2 min; ethanol 70% 2 min; ethanol 50% 2 min and DEPC Water 2 times, 2 min) and treated at 37ºC for 35 min with proteinase K (0.1 $U.mL^{-1}$). After blocking the proteinase K, sections were washed for 2 min in PBS containing 0.2% glycine and twice with PBS. Sections were dehydrated with graded ethanol series (ethanol 50% 1 min, ethanol 70% 1 min and ethanol 100% twice 1 min) and air-dried. Hybridizations were carried out in a humid chamber at 45 ºC overnight using 600 ng of the corresponding probe, as described in Jabnoune et al. (2009). Non-linked probes were removed with 20 μgmL^{-1} RNase A for 30 min at 37 ºC. Immunological detection of digoxigenin-labelled RNA hybrids was performed with anti-digoxigenin antibodies conjugated with alkaline phosphatase enzyme (Roche), following manufacturer´s instructions. Finally, blue staining was performed using Vector Blue Alkaline Phosphatase Substrate kit (Vector Laboratories) according manufacturer´s instructions and images were taken on the Nikon Eclipse

Ni-E microscope (Nikon Corporation, Tokyo, Japan). An aliquot of the same root fragments was separated to estimate mycorrhizal colonization.

Sequence analyses

The *RiCRD* gene sequence was previously identified by Tamayo et al. (2014) in *R. irregularis* genome available in the JGI website (https://genome.jgi.doe.gov/portal/). Transmembrane domains of the protein were predicted using the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The E1-E2 ATPase, Hydrolase and Heavy Metal associated domains (HMA) were identified via the Pfam Software v. 32.0 (https://pfam.xfam.org/). These results were used to generate a structural model of RiCRD using MyDomains tool of Prosite (https://prosite.expasy.org/mydomains/). Protein subcellular localization was predicted by WoLF PSORT (https://wolfpsort.hgc.jp/).

Statistical analyses

Statistical analyses were performed with IBM SPSS Statistic software v.25. Data was subjected to the Student´s t-test when two means were compared or by one-way ANOVA using Post hoc comparison with the Tukey-B test to find out differences among groups of means. Results were accepted as significant at $P < 0.05$. The data are expressed as mean +/- standard error. All the analyses are based on at least 3 biological replicates per each treatment ($n \geq 3$).

Results

Sequence analyses of the **Rhizophagus irregularis** *RiCRD heavy metal ATPase*

The full-length cDNA sequence of *RiCRD* encodes a protein of 946 amino acids that contains all the characteristic features of P_{1B}-type (CPx-type) ATPases, including the conserved transmembrane cysteine-proline-cysteine motif (CPC) that is essential for metal translocation. The protein contains eight transmembrane helices with the CPC motif located in the sixth transmembrane helix, the N- and C-termini facing the cytoplasmic side, two heavy metal associated domains (HMA; PF00403) in the N-terminus, the E1-E2 ATPase domain (PF00122), the hydrolase domain (PF00702) including the DKTGT phosphorylation signature sequence and the invariant histidine-proline HP dipeptide at 41 residues C- terminal from the phosphorylation signature (Solioz and Vulpe, 1996; Arguello et al., 2007; Smith et al., 2014) (**Figure 1**). RiCRD was predicted to be located at the plasma membrane.

Comparisons of the full-length cDNA with the genomic sequence revealed the presence of two introns of 92 and 76 bp flanked by the characteristic splicing sequences GT and AG at the 5’ and 3’ ends, respectively (**Figure 1**).

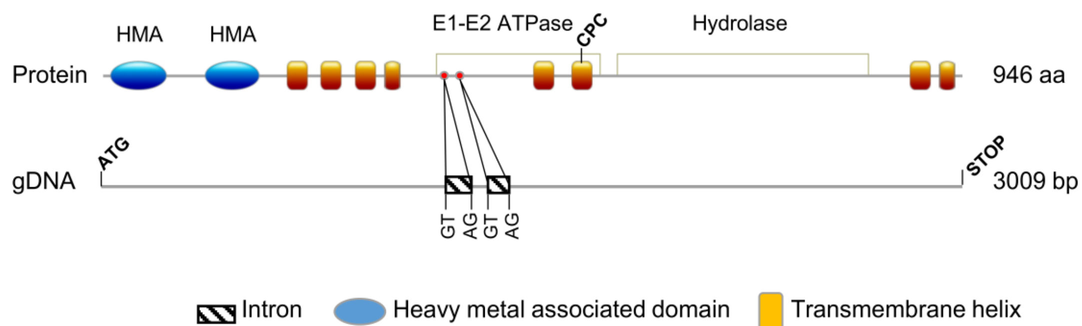

Figure 1. Schematic representation of the structure of *R. irregularis* RiCRD despicting the position of characteristic features of P1-type ATPases. This model was generated with MyDomains tool of Prosite (https://prosite.expasy.org/mydomains/) based on the results of the TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and the Pfam Software v. 32.0 (https://pfam.xfam.org/) (upper panel). Exon/ intron organization of the *RiCRD* genomic sequence, introns were illustrated with striped boxes and flanked by the canonical splicing sequences GT an AG at 5' and 3' ends, respectively (lower panel).

RiCRD *is more highly expressed in the intraradical mycelium*

As a first step towards understanding the role that *RiCRD* could play in *R. irregularis*, we assessed its expression level in the ERM and IRM collected from the *in vitro* (monoxenic cultures) and *in vivo* (sandwich system) experimental systems used to grow the AM fungus. Transcript levels of the *R. irregularis* high-affinity monosaccharide transporter *RiMST2*, which is strongly up-regulated in the IRM during AM symbiosis (Helber et al., 2011), was also determined as a marker of fungal activity. Carrot roots collected from the monoxenic cultures presented 10 % of mycorrhizal colonization while the percentage of mycorrhizal colonization of the chicory roots used to grow the fungus in the sandwich system was 78 %. In both experimental systems, *RiCRD* was more highly expressed in the IRM than in the ERM. *RiCRD* transcript levels were 18-fold higher in the carrot mycorrhizal roots than in the ERM collected from the monoxenic cultures and 25-fold higher in the mycorrhizal chicory roots than in the ERM collected from the *in vivo* sandwich system (**Figure 2**). This expression pattern suggests that RiCRD might account for Cu efflux from the fungus to the apoplast of the symbiotic interface.

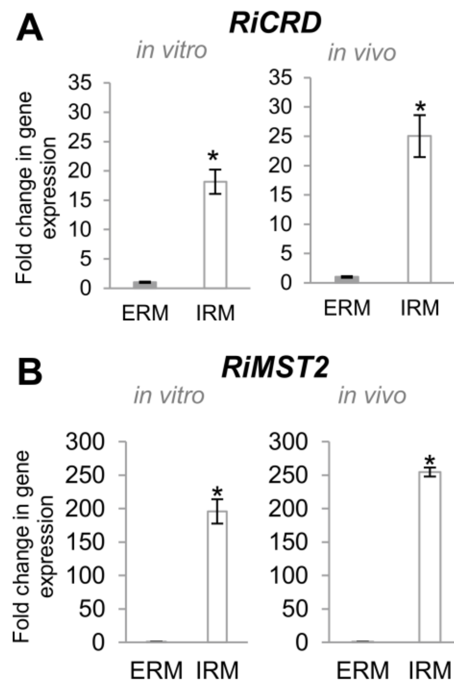

Figure 2. Expression levels of *RiCRD* (**A**) and *RiMST2* (**B**) in the extraradical mycelia (ERM) and the intrarradical mycelia (IRM) of *R. irregularis* grown under control conditions in monoxenic cultures with T-DNA transformed carrot roots (*in vitro* system) or in the whole plant bidimensional experimental system with chicory plants (*in vivo* system). Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using the *RiEF1α* as a normalizer; the expression levels of the corresponding gene in the ERM was designated as 1. Bars represent standard error; * $P < 0.05$ statistically significant differences.

RiCRD ***is expressed in the arbuscules***

Given that arbuscules developed in cortical cells are the main structure where nutrient exchange between symbionts take place, Cu transfer from the fungus to the plant should occur in the arbuscule-colonized cortical cells (Luginbuehl and Oldroyd, 2017; MacLean et al., 2017). However, since the fungus develops other intraradical structures, we decided to determine the specific fungal structure where *RiCRD* is expressed by performing an *in situ* hybridization assay in tomato roots presenting a 40 % of mycorrhizal colonization (**Figure 3A-B**). As a positive control of hybridization and RNA quality, expression of the 18S ribosomal gene was also monitored. *RiCRD* transcripts were clearly detected in the arbuscules developed in the inner cortical while we did not find signal in other kind of fungal structures, indicating that arbuscules are likely the sites of Cu efflux (**Figure 3C-F**).

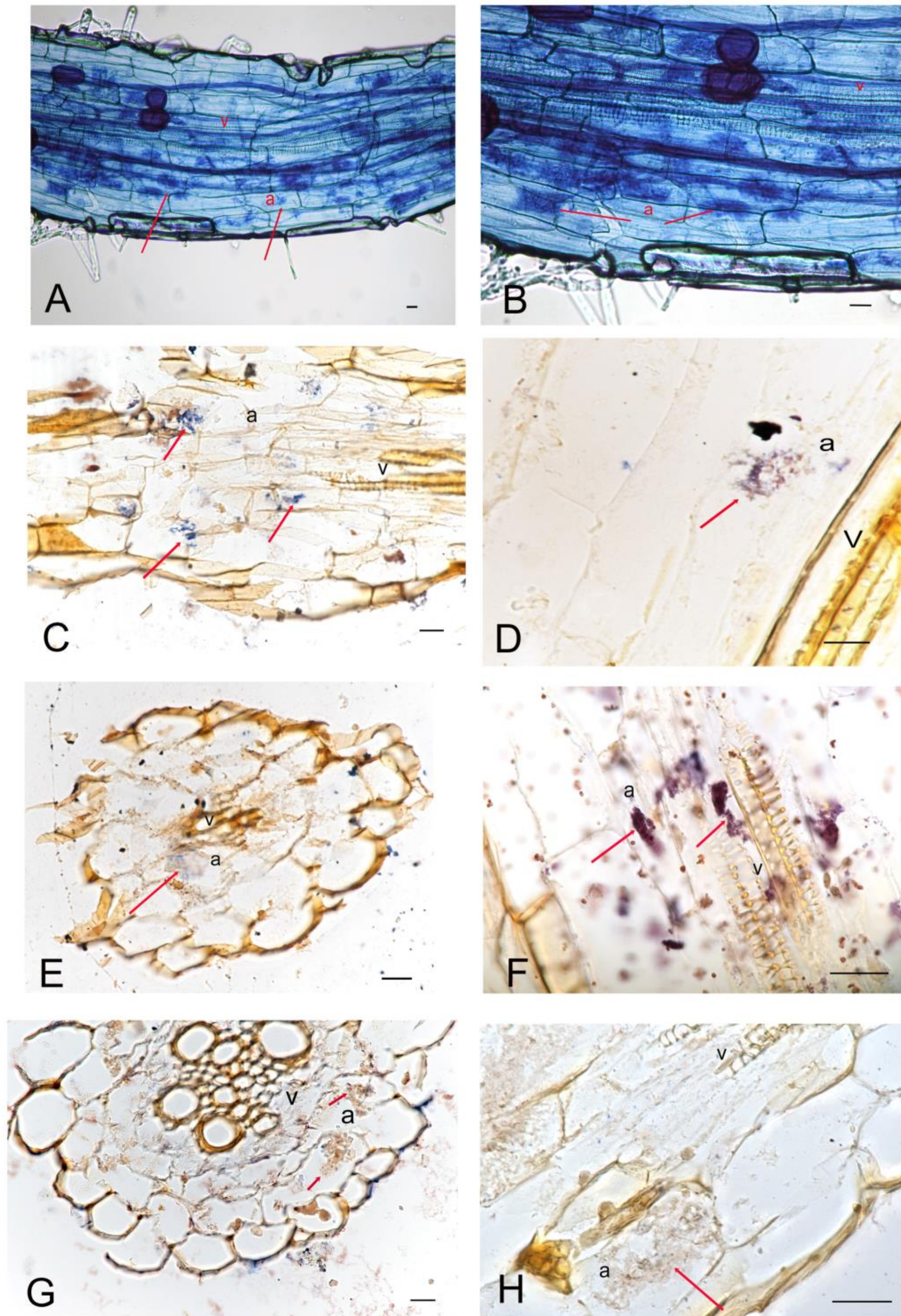

Figure 3. Localization of RiCRD transcipts by *in situ* hybridation in mycorrhizal tomato roots (*L. esculentum* cv. Moneymaker). (**A-B**) Tripan blue staining of roots (**C-F**) hybridation with *RiCRD* antisense probe showing positive blue stanining in arbuscules (**G-H**) hybridation with *RiCRD* sense probe in which only a weak background signal was detected. a: arbuscles (see red arrows), v: vascular tissues. Scale bars represent 20µm

Expression of RiCRD in planta *decreases in Cu-deprived roots*

To test whether Cu transfer from the fungus to the plant is dependent on Cu availability; we assessed the influence of growing the roots under Cu-limiting conditions on the transcription of *RiCRD*. For this purpose, expression of *RiCRD* was determined by qRT-PCR in carrot roots grown in monoxenic cultures in M media with 0.5 µM Cu (control) or in M media without Cu (Cu deficiency) and in chicory roots grown in the sandwich system and fertilized with either half-strength Hoagland nutrient solution containing 0.16 µM Cu (control) or without Cu (Cu deficiency). Cu deficiency decreased mycorrhizal colonization, which was confirmed molecularly by the quantification of the amount of the fungus within the root (data not shown). Expression of *RiCRD* was down-regulated in the Cu-deprived carrot and chicory roots, although to a higher extent in the carrot roots grown in monoxenic cultures (**Figure 4**). These data suggest that Cu efflux from the fungus is reduced under Cu-limiting conditions.

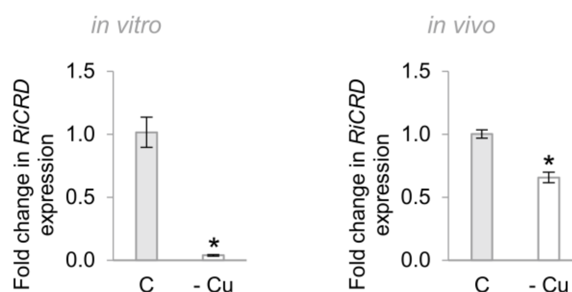

Figure 4. Effect of Cu deficiency on *RiCRD* expression during the *in planta* phase of the AM fungus *R. irregularis.* *RiCRD* expression in mycorrhizal carrot roots carrot roots developed in monoxenic cultures in M media (control, 0.5µM Cu) or in M media lacking Cu (left panel), and in mycorrhizal chicory roots grown in the whole plant bidimensional experimental system and fertilized with 0.5X Hoagland solution (control, 0.16µM Cu) or with a modified nutrient solution without Cu (right panel). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method using the *RiEF1α* as a normalizer. Bars represent standard error; * $P < 0.05$ statistically significant in comparison to the corresponding control value.

RiCRD *is involved in Cu detoxification*

To investigate whether RiCRD could play a role in fungal Cu tolerance by detoxifying Cu excess out of the fungus, *RiCRD* gene expression was assessed by real time quantitative RT-PCR (qRT-PCR) in ERM grown in monoxenic cultures under different Cu levels. As previously observed by Cornejo et al. (2013), some blue spores indicative of Cu compartmentalization were observed 2 days after Cu addition to the ERM while *RiCRD* expression was down-regulated in the ERM grown under Cu deficiency, exposure of the mycelia to high Cu levels triggered transcription of *RiCRD* at all the time points analyzed (**Figure 5A**). The highest transcripts accumulation (29-fold induction) was reached 2 days after exposure to 500 µM $CuSO_4$. These results are consistent with a role of RiCRD on Cu detoxification.

RiCRD *is involved in cadmium detoxification*

Since some Cu-ATPases can transport other metal ions, we also tested whether RiCRD could be involved in Cd detoxification by determining *RiCRD* transcript levels in the ERM grown monoxenically and exposed to 45µM of $CdSO_4$ for different periods of time. Interestingly, *RiCRD* expression was transiently induced by cadmium toxicity. A 3-fold induction was observed 3 and 6 h after Cd addition (**Figure 5B**).

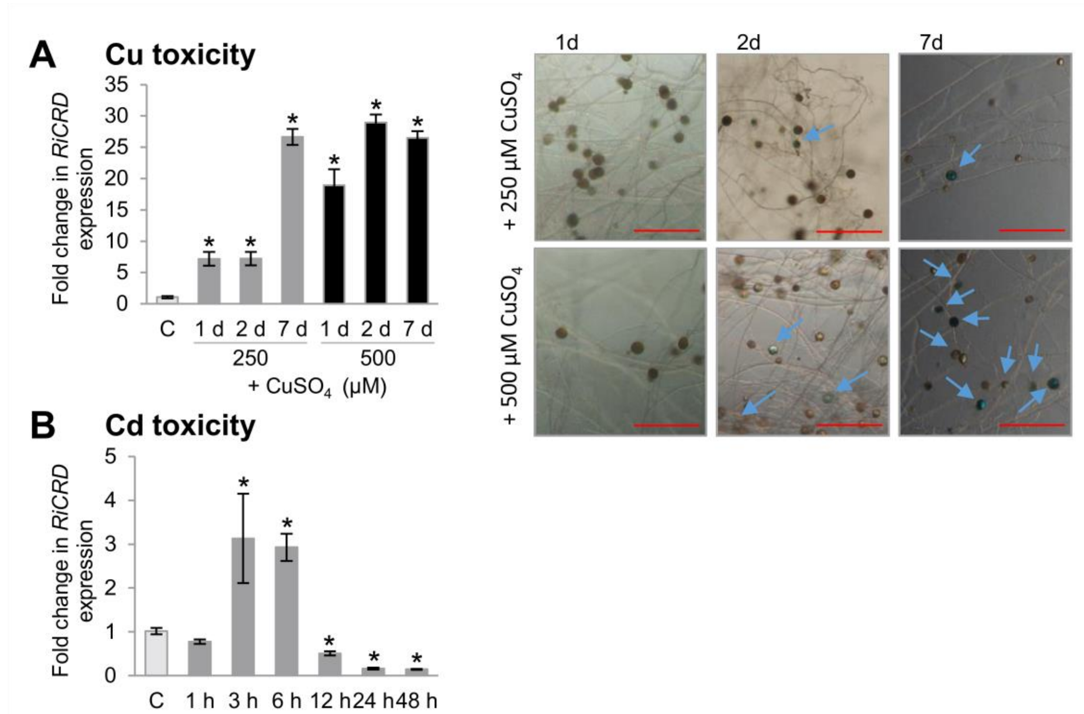

Figure 5. Efect of metal toxicity on *RiCRD* ERM expression. *R. irregularis* ERM was grown in monoxenic cultures in M-C medium (control) or in M-C medium supplemented with 250 µM CuSO4, 500 µM CuSO4 **(A)** or with 45 µM CdSO4 **(B)** and incubated at 24°C.The time of medium exchange was referred as time 0. Mycelia were collected 1, 2 and 7 days after Cu addition and 1, 3, 6, 12, 24 and 48 hours after Cd supplementation. Some blue spores indicative of Cu compartmentalization were observed 2 days after Cu addition to the ERM; images were captured under a binocular microscope just before collect the ERMs subjected to the different Cu treatments (Scale bar: 500 µm). *RiCRD* expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using the *RiEF1α* as a normalizer. Bars represent standard error; * $P < 0.05$ statistically significant in comparison to the corresponding control value.

RiCRD is a major player in R. irregularis *Cu tolerance*

To get some clues about the significance of RiCRD on metal tolerance in *R. irregularis*, we have compared its expression levels in the Cu- and Cd-treated ERM with the expression levels of *RiMT* and *RiABC*, genes that have been previously shown to be involved in metal detoxification in *R. irregularis*. *RiMT* (formerly named *GintMT*) encodes a metallothionein, a cysteine-rich low molecular mass polypeptide with high affinity for metals (González-Guerrero et al., 2007) and *RiABC1* (formerly named *GintABC1*) an ABC type-transporter that was suggested to mediate

transport of metal-glutathione or metal-phytochelatin complexes to the vacuoles (González-Guerrero et al., 2010). Compared to the expression levels in the control ERM, a 2-fold induction in *RiMT* transcript levels was detected when the ERM was exposed 2 days to 500µM $CuSO_4$. No changes on *RiMT* expression were detected in the Cd-treated ERM (**Figure 6**). Additionally, a 2-fold induction in *RiABC* was detected when the ERM was exposed 2 days and 7 days to 500µM $CuSO_4$ whereas *RiABC* was 5-fold transiently induced 6h after Cd supplementation (data not shown).

The higher induction of *RiCRD* expression in response to Cu exposure than of the other regulators of the intracellular metal levels suggests that metal efflux pumps are major determinants of Cu tolerance in *R. irregularis*.

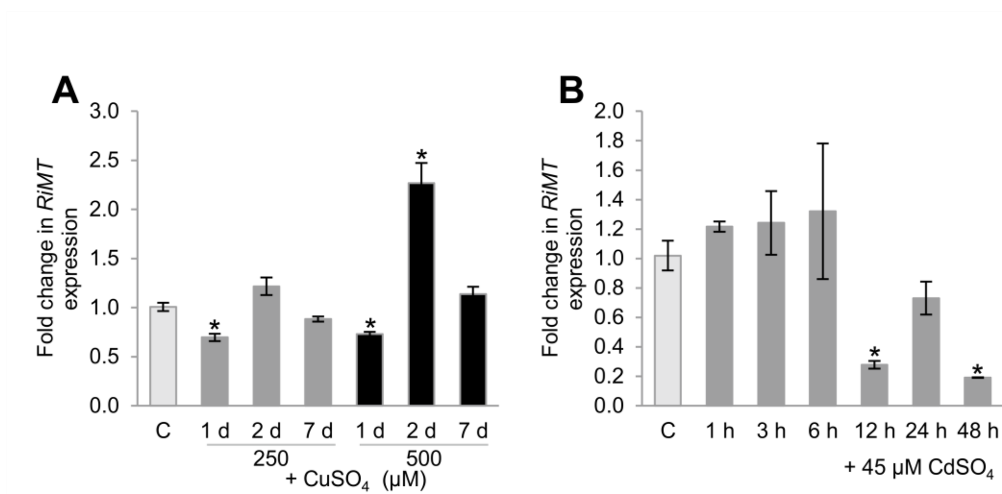

Figure 6. Effect of metal toxicity on *R. irregularis* metallothionein expression (*RiMT*). *R. irregularis* ERM was grown in monoxenic cultures in M-C medium (control) or in M-C medium supplemented with 250 µM CuSO4, 500 µM CuSO4 (**A**) or with 45 µM CdSO4 (**B**) and incubated at 24°C.The time of medium exchange was referred as time 0. Mycelia were collected 1, 2 and 7 days after Cu addition and 1, 3, 6, 12, 24 and 48 hours after Cd supplementation. Gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using the *RiEF1α* as a normalizer. Bars represent standard error; * $P < 0.05$ statistically significant in comparison to the corresponding control value.

Discussion

AM fungi play an important role in modulating plant Cu acquisition in a wide range of Cu concentrations. The potential of AM fungi, on the one hand, to increase Cu uptake when plants are grown in low Cu soils and, on the other hand, to alleviate Cu toxicity has led to the hypothesis that AM function as a “buffer” to protect the plant against damage caused by Cu in the soil (Ferrol et al., 2016). Here, we report characterization of *RiCRD*, a *R. irregularis* gene putatively encoding a Cu-ATPase with a dual function in Cu transfer to the host plant in the IRM and in Cu tolerance in the ERM.

In silico analysis of the RiCRD protein and expression patterns of *RiCRD* when the ERM was exposed to high Cu and Cd levels strongly suggest that RiCRD is the ortholog of CaCRD1, a plasma membrane P_{1B} type ATPase which confers high resistance of toxic Cu levels to the pathogenic yeast *Candida albicans* acting as an efflux pump (Riggle and Kumamoto, 2000; Weissman et al., 2000). RiCRD has all the characteristic features of P_{1B} type ATPases, containing eight transmembrane domains, with the CPC motif that is needed for metal translocation in the sixth transmembrane helix (Arguello et al., 2007). Additionally, the invariant HP dipeptide was found 40 residues downstream to the phosphorylation site. Although the function of this motif is still unknown, it seems to have some relevance since a change of this histidine for a glutamic acid in Wilson disease protein renders abnormalities on copper metabolism (Tanzi et al., 1993; Solioz and Vulpe, 1996; Bissig et al., 2001). Interestingly, RiCRD has two heavy metal associated domains (HMA) in the N-terminus, although only one strictly has the classical GMXCXXC motif. The first domain, GLTCASC, has the CXXC motif characteristic of proteins that bind copper (Camakaris et al., 1999; Strausak et al., 1999; Smith et al., 2014; Migocka, 2015), but the second methionine is changed by a leucine. The N-terminus of RiCRD presents a reduced number of metal binding domains in comparison with other eukaryote Cu-ATPases, which usually have multiple repeats of this domain (Rensing et al., 1999; Arguello et al., 2007). For instance, its CaCRD ortholog has five metal binding domains, including two CXXC motifs and three GMXCXXC signatures (Riggle and Kumamoto, 2000; Weissman et al., 2000). These small differences found in the RiCRD sequence could be linked with a singular function of the protein involving alternative regulation mechanisms or targeting (Arguello et al.2007). On the other hand, RiCRD was predicted to be localized in the plasma membrane supporting a role in metal efflux from the cytosol as its *C. albicans* ortholog (Riggle and Kumamoto, 2000; Weissman et al., 2000).

Regarding the gene expression analysis, the finding that *RiCRD* expression in the ERM was strongly up-regulated by Cu toxicity indicates that RiCRD plays a role in copper detoxification. Increased levels of RiCRD would enable the fungus to handle the excess of Cu by facilitating its efflux through the plasma membrane and avoiding the accumulation of intracellular Cu toxic levels. High eukaryotes usually cope with metal toxicity by chelating metals in the cytoplasm through small cysteine-rich peptides called metallotioneins, whereas a metal efflux based-strategy is more common in prokaryotes (Hamer, 1986; Silver, 1996). Our data showing that *RiMT* expression was just slightly and transiently induced in the ERM subjected to the highest Cu concentrations suggests that *R. irregularis* uses the Cu efflux RiCRD pump as primary mechanism to overcome copper toxicity rather than a Cu chelation strategy by metallothioneins, as it has been previously described in *Candida albicans* and *Aspergillus nidulans* (Riggle and Kumamoto, 2000; Weissman et al., 2000; Antsotegi-Uskola et al., 2017). In fact, the role of the *R. irregularis* metallothionein *RiMT* in metal tolerance was attributed to its antioxidant activity

against the metal-induced oxidative stress rather than on its metal chelation activity (Gonzalez-Guerrero et al., 2007). This in contrast with what happens in *Saccharomyces cerevisiae,* in which Cu resistance relies mainly in Cu chelation by the CUP1 metallothionein (Ecker et al., 1986; Thiele, 1988). In addition to this Cu efflux strategy, as previously reported by Cornejo et al., (2013), *R. irregularis* compartmentalizes part of the excess Cu in some spores of the fungal colony, as some blue spores indicative of Cu accumulation were observed in some of the Cu-exposed ERM.

RiCRD expression was additionally up-regulated by cadmium toxicity, although in this case its induction was faster, transient and less intense than with copper. We hypothesize that RiCRD can transport both metal ions, although copper is probably its principal substrate. Since cadmium is much more toxic than copper, cells might require a faster induction of *RiCRD* as a first step to avoid cadmium toxicity. Cu exporting P-type ATPases from other organisms, including fungi, have been shown to transport different metals (Rensing et al., 1999; Gatti et al., 2000; Riggle and Kumamoto, 2000; Antsotegi-Uskola et al., 2017; Benes et al., 2018). In *S. cerevisiae*, Cd efflux occurs through the cadmium-specific pump PCA1, belonging also to the P_{1B} type ATPase subfamily but being phylogenetically separated from the CRD-like ATPases (Adle et al., 2007; Saitoh et al., 2009). However, the *R. irregularis* genome lacks PCA1-like orthologs (Tamayo et al., 2014). Although the first response to Cd exposure could be activation of RiCRD to transport out of the cytosol excess Cd, *R. irregularis* must activate other mechanisms for Cd detoxification. The finding that *RiMT* expression was not affected by Cd, supports the hypothesis that cadmium detoxification should relay in other more specific players (Gonzalez-Guerrero et al., 2007), such as phytochelatins, small peptides enzymatically synthetized from glutathione which form complexes with metals mainly cadmium in the cytoplasm to be lately sequestered into vacuoles (Cobbett, 2000; Heiss et al., 2003; Mendoza-Cozatl et al., 2010). This hypothesis agrees with up-regulation by Cd of *RiABC*, a gene putatively encoding a transporter that mediates transport of metal-glutathione or metal-phytochelatin complexes to the vacuoles (González-Guerrero et al., 2010). Shine et al. (2015) revealed the presence of a putative phytochelatin synthase in *R. irregularis* genome although its contribution to cadmium resistance remains to be elucidated.

In this manuscript we describe for the first time that AM fungi use a Cu efflux strategy to cope with Cu toxicity. Therefore, identification of RiCRD as the primary strategy to overcome Cu toxicity is the special relevance since the only example of heavy metal efflux as an adapted tolerance mechanism in AM fungi is an arsenate pump, which acts coordinately with a high affinity phosphate transporter (Gonzalez-Chavez Mdel et al., 2011).

On the other hand, remarkably, RiCRD was more highly expressed in the intraradical mycelia than in the extraradical mycelia isolated form *in vitro* and *in vivo* cultures of *R. irregularis*. Joining the fact that RiCRD is localized at the plasma membrane acting as an extrusion pump of copper and that is highly expressed during the *in planta* phase of the fungus, it is tempting to propose that RiCRD might be the transporter responsible to supply copper from the fungus to the plant in the symbiotic interphase. This is also supported by the finding of *RiCRD* transcripts in arbuscules contained in the inner cortical cells of mycorrhizal tomato roots by *in situ* hybridization. Furthermore, repression of *RiCRD* transcripts during the *in planta* phase by Cu scarcity indicates that the fungus could reduce the efflux of copper to the symbiotic interface to satisfy its own needs when Cu is limited in the environment. In this interesting scenario, the fungal partner could exert some control on the efflux of copper to the plant partner by the regulation of *RiCRD* transcripts. This is consistent with previous studies in which a nutritional competition between both symbionts have been proposed in function of nutrients and micronutrients availability (Balestrini et al., 2007; Kiers et al., 2011; Pérez-Tienda et al., 2011; Tamayo et al., 2018).

In conclusion, data presented in this work shown that *R irregularis* copper resistance mainly relies on a Cu exporting P-Type ATPase which could have a big impact not only in copper detoxification pathway but also on the delivery of copper from the fungus to the plant in the symbiotic interface being a crucial player for copper homeostasis in AM symbiosis. This is the first Cu efflux strategy to overcome metal excess characterized in AM fungi. Although this study provides a breakthrough in the knowledge about copper homeostasis in AM fungi, further studies are necessary to completely understand this complex Cu homeostatic network, which allow AM fungi to maintain Cu intracellular levels balanced in a wide range of environments.

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Chapter IV: Arbuscular mycorrhiza modifies the physiological plant response to Cu toxicity and HMA gene expression patterns in Zea mays

Introduction

Cu is an essential micronutrient for normal plant metabolism, acting as a cofactor in numerous physiological processes, such as photosynthesis, mitochondrial respiration, superoxide scavenging, and cell wall metabolism (Hänsch and Mendel, 2009). However, at high concentrations is toxic. As a redox-active metal, excess Cu generates various reactive oxygen species via its participation in Haber-Weiss and Fenton reactions, which in turn produce an oxidative stress that negatively affects plant growth and development (Linder, 1991). Toxicity can also result from displacement of other essential metals or from its binding to the sulfhydryl groups of proteins, leading to the inhibition of their activity or disruption of their structure (Macomber and Imlay, 2009).

Although Cu is a trace element that makes up only 0.00007% of the Earth's crust, the excessive use of Cu pesticides and fungicides, the release of industrial wastewater and waste deposited from smelting and mining activities have led to Cu contamination in many arable soils around the globe. Excessive soil Cu accumulation is not only toxic to plants, but also produces health hazards to people and animals through the food-chain given the inhibition of crop biomass and yield and the effect of metal accumulation in the plants.

Plants have evolved certain strategies to avoid this toxicity, which include reduction of Cu uptake, stimulation of Cu efflux through the plasma membrane, chelation by metallothioneins or phytochelatins, sequestration in the vacuoles and activation of antioxidant systems. In addition to these constitutive strategies, associations with some beneficial rhizosphere microorganisms also contribute to plant Cu tolerance. Among them, arbuscular mycorrhizal (AM) fungi have received special attention because of their role in the improvement of plant nutrition and tolerance to several environmental stresses (Ferrol et al, 2016).

AM fungi are obligate biotrophs that form mutualistic symbiosis with 72% of vascular plants, including several crops (Brundrett and Tedersoo, 2018). As obligate biotrophs, AM fungi require the supply of carbon compounds from the plant, either in the form of carbohydrates or lipids for the development and function of mycorrhizal structures (Helber et al., 2011; Jiang et al., 2017). In return, the fungus provides the plant with low mobility mineral nutrients and water (Lanfranco et al., 2018). Moreover, development of the symbiosis alleviates in the host plant the deleterious effects of biotic and abiotic stresses, such as salinity, drought and metal toxicity (Jung et al., 2012; Ferrol et al., 2016; Quiroga et al., 2019).

Alleviation of plant metal toxicity by AM fungi depends on the metal and on the fungal and plant species involved in the association (Weissenhorn et al., 1995; Liao et al., 2003). The mechanisms underlying the beneficial effect of AMF on plant fitness in metal contaminated soils are diverse. Metal toxicity alleviation by AM fungi could be due to a reduced metal uptake or lower root to shoot partitioning as a consequence of metal immobilisation by the structures the

fungus develops in the soil or in the root, to the increased plant mineral nutrition and development of the mycorrhizal plant or to the modulation of the plant metal response (Ferrol et al., 2016). Proteomic and transcriptomic untargeted approaches showed that the plant responses induced by the metal were absent or inverse in mycorrhizal roots (Repetto et al., 2003; Aloui et al., 2009; Pallara et al., 2013). Several studies have also analysed the effect of the AM symbiosis on the plant intrinsic mechanisms of Cu tolerance, such as metallothioneins, phytochelatins and activity of antioxidant enzymes (Cicatelli et al., 2010; Pallara et al., 2013; Merlos et al., 2016). However, nothing is known about the effect of AM on the regulation of the processes that control export of Cu excess out of the cytosol.

Two major transporter families are involved in Cu transport, the COPT-CTR family and the P_{1B}-ATPases. Plant Cu acquisition is governed by transporters of the COPT-CTR family, while Cu efflux is mediated by P_{1B}-type ATPases (Puig, 2014; Migocka, 2015). These ATPases, known as HMAs (Heavy Metal ATPases) belong to the P-type ATPase superfamily and use the energy provided by ATP hydrolysis to pump metals across membranes against their electrochemical gradient. Structurally, they are characterized to possess typically six or eight transmembrane domains with a CPX/SPC motif contained in the sixth transmembrane helix and metal binding domains at the N and/or C terminus (Arguello et al., 2007). Higher plants have more HMAs than other organisms, with a wide variety of functions (Williams and Mills, 2005). They have been largely studied in the dicotyledonous model plant *Arabidopsis thaliana*, with their physiological roles well described. The *Arabidopsis thaliana* HMA family comprises eight members: AtHMA1 to AtHMA8. Among them, five HMAs have been related with Cu transport: AtHMA5, AtHMA6 (also known as PAA1), AtHMA7 (also known as RAN1) and AtHMA8 (also known as PAA2) belonging to the subgroup P_{1B-1}which, which are highly specific for Cu^{+}/Ag+ transport, and AtHMA1 belonging to the subgroup P_{1B-4}, which includes transporters with broad-specificity of divalent metal ions, including Cu. However, the roles of the different HMAs in monocotyledonous plants still poorly understood.

The model monocotyledonous plant *Oryza sativa* has nine HMA genes (OsHMA1 to OsHMA9), but the function of most of them is largely unknown. With the aim of getting insights into the role of the metal efflux transporters in Cu detoxification in maize plants and on their regulation by the AM symbiosis, we have searched the maize genome for genes putatively encoding heavy metal ATPases and determined their expression patterns in non-mycorrhizal and mycorrhizal plants grown under different Cu supplies. Here, we report how inoculation of maize plants with the AM fungus *R. irregularis* modifies the physiological plant response to Cu toxicity and HMA gene expression.

Materials and Methods

Biological materials and growth conditions

The Cu sensitive cultivar Orense of *Zea mays* L. (Madejón et al., 2009) and the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler DAOM 197198 were used in this study. The fungal isolate was maintained in open-pot trap cultures established with *Trifolium repens* L. and *Sorghum vulgare* L. plants. The inoculum was obtained in a sepiolite -vermiculite substrate and contained a mixture of mycelia, spores and mycorrhizal root fragments.

Maize seeds provided by Semillas Fitó (Barcelona, Spain) were surface-sterilized and germinated for 3 days at 28 °C in a Petri dish containing a sterile wet filter paper. Seedlings were transferred into 1 L pots containing a steam-sterilized soil mixed with peat (3 %) at pH 6 supplemented with different Cu concentrations and 10% of the sepiolite -vermiculite based inoculum of *R. irregularis* (mycorrhizal plants, MYC) or into pots containing the growth substrate and 10 % of the substrate used to obtain the inoculum (non-mycorrhizal plants, NM). Non-mycorrhizal plants received a filtrate of the AM fungal inoculum (< 20 µm) to provide the non-mycorrhizal soil microbiota.

Soil was collected from Güejar Sierra, Granada, Spain and had a pH of 6.58 [measured in water 1:5 (w/v)], 10.84% organic matter, 0.47% total N, 0.03% total P, 0.38% Ca and 0.42% K. Supplementation of soil with different Cu concentrations (0, 100, 250 mg Cu kg−1 soil) were performed by adding a $CuSO_4$ liquid solution, mixing thoroughly and allowing to stabilize for 15 days.

Plants were grown during 5 weeks in a growth chamber at 25ºC/18ºC day/night and 16 h light photoperiod and watered three times per week with 25 ml H_2O. After harvesting, plant biomass was evaluated by measuring root and shoot fresh weights. Roots and shoots of all plants were frozen in liquid nitrogen and stored at -80 °C until use.

Experimental design

A full factorial design with two factors: mycorrhization (M) and copper availability (Cu) was performed. The mycorrhizal factor consisted in two levels (non-mycorrhiza NM; and mycorrhizal inoculated plants MYC) and the copper factor had three levels (0, 100, 250 mg Cu kg−1). Seven replicates were considered, giving a total of 21 pots. Three pools of two plants each were considered for nutrient determination and gene expression analysis.

Mycorrhizal colonization

Mycorrhizal colonization was estimated in root samples after clearing with KOH (10%) and trypan blue staining (0.05%) (Phillips and Hayman, 1970). Arbuscular colonization (AC %) was estimated using the magnified intersections method, as described by McGONIGLE et al., (1990).The abundance of AM fungus in the roots was also determined molecularly by analysing the expression levels of the *R. irregularis* elongation factor 1α (*RiEF1α*; GenBank Accession No. DQ282611). Additionally, the expression levels of the mycorrhiza-induced phosphate transporter of *Z. mays* ZmPht1;6 (GenBank Accession No. AY974046) was also analysed as an indicator of a functional AM symbiosis in maize roots (Nagy et al., 2006).

Determination of mineral nutrients in plant tissues

Shoots and roots were oven dried at 70 °C for 48 h, and ground to a fine powder for nutrient content determinations. Nutrients were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP 6500 Duo Thermo), using the Technical Service of the Estación Experimental del Zaidin, EEZ-CSIC from Granada, Spain. Nutrient data were subjected to a sparse partial least-squares discriminant analysis (sPLSDA); for this purpose, data were previously transformed with generalized logarithm transformation and scaled by the pareto method by using the METABOANALYST web-based metabolomic package (https://www.metaboanalyst.ca/).

Identification of maize HMAs and sequence analyses

Nucleotide sequences of *Z. mays* HMAs were downloaded from the Aramemnon plant membrane protein database 8.1 (http://aramemnon.uni-koeln.de/) and used as a query for additional BLASTx searches in the *Z. mays* genome deposited on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Maize Genetics and Genomics (https://www.maizegdb.org/) and Gramene (http://gramene.org/) websites. Full-length amino acid sequences were aligned with the orthologous sequences of *Arabidopsis thaliana* and *Oryza sativa* HMAs via Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Alignments were imported to the Molecular Evolutionary Genetics Analysis software v. 6 (MEGA) to generate a phylogenetic tree via the Neighbor-Joining method with 1000 bootstrap replicates, using the Poisson model and pairwise deletion of gaps options for distance computation. ZmHMAs subcellular location was predicted by WoLF PSORT I/ II (https://wolfpsort.hgc.jp/; https://www.genscript.com/wolf-psort.html) and TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/). Predictions of putative transmembrane helices were carried out with TOPCONS web server (http://topcons.cbr.su.se/).

Gene expression analysis

Total RNA was extracted from plant tissues using the phenol/SDS method followed by LiCl precipitation as described by Kay et al. (1987) and DNase treated with the RNA-free DNase set (Qiagen) according to the manufacturer's instructions. Quantification of isolated RNAs was carried out with the Nanodrop 1000 Spectrophotometer (Thermo Scientific) and 1 µg of each RNA was used for the cDNA synthesis in a 20 µL final volume reaction containing 200 U of SuperScript III Reverse Transcriptase (Invitrogen) and 2.5µM oligo $(dT)_{20}$ primer (Invitrogen), following the manufacturer's instructions. Transcript levels of genes were determined in an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad) in a 11 µL final volume reaction containing 1 µL of a 1:10 dilution of cDNA template, 5.5 µL of SYBR® Premix Ex Taq™ (Tli RNaseH Plus), Bulk 2X (Takara) and 0.2µM of the corresponding specific primers. All the determinations were performed in at least three biological samples with the threshold cycle (Ct) determined in duplicate and in at least two independent PCRs experiments. The amplification protocol used was 95ºC for 30s, 38 cycles of [95ºC for 5s, 60ºC for 30s, 72 ºC for 30s] followed by a dissociation stage, except for *ZmHMA5.1* in which the annealing step was carried out at 63 ºC to avoid nonspecific amplifications. Abundance of transcripts was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized with the expression levels of the elongation factor 1α of *Z. mays* (*ZmEF1α*, GenBank Accession No. NM_001112117).

Statistical analyses

Statistical analyses were performed through IBM SPSS Statistic software v.23. Data were subjected to a two-way ANOVA, considering Mycorrhization (M) and copper availability (Cu) as factors. Post hoc comparisons were evaluated using the Tukey-B test to find out differences among groups of means ($P < 0.05$), when necessary. All the analyses are based on at least three biological replicates per each treatment ($n \geq 3$).

Results

Plant growth and mycorrhizal colonization

Shoot and root fresh weights decreased with increasing soil Cu concentrations in non-mycorrhizal and mycorrhizal plants. However, this decrease was higher in non-mycorrhizal plants. At all Cu concentrations assayed, growth of mycorrhizal plants was higher than growth of non-mycorrhizal plants (**Table 1**).

Table 1. Effect of Cu toxicity and mycorrhizal inoculation on maize biomass. Shoot and root fresh weight (g) in non-mycorrhizal (NM) and mycorrhizal inoculated maize plants (MYC) grown in Cu supplemented soils with 0, 100, 250 mg Cu soil^{-1}. Values are means ± standard error. Different letters show statistically significant differences ($P < 0.05$) among treatments.

	0 (mg kg^{-1} soil)		100 (mg kg^{-1} soil)		250 (mg kg^{-1} soil)	
	NM	**MYC**	**NM**	**MYC**	**NM**	**MYC**
SHOOT	11.45±0.55 **bc**	14.9±0.38 **a**	8.45±0.48 **d**	12.98±0.37 **b**	5.34±0.28 **e**	9.74±0.43 **cd**
ROOT	5.78±0.46 **bc**	7±0.38 **ab**	4.84±0.34 **c**	7.67±0.37 **a**	3.24±0.19 **d**	5.6±0.21 **c**

Fungal colonization decreased when plants were gown in a soil supplemented with 250 mg Kg-1 soil. Interestingly, while arbuscule frequency was not affected in roots grown in a soil supplemented with 100 mg Kg-1 soil, expression of *ZmPht1;6*, a marker of arbuscule activity, was lower in roots grown at 100 mg Kg-1 soil than on the control roots (**Figure1**).

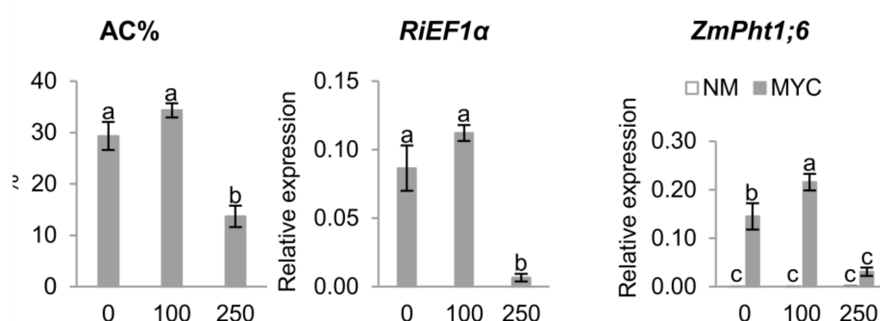

Figure 1. Effect of Cu toxicity on mycorrhizal colonization. Arbuscular colonization (AC%) was assessed in root samples after trypan blue staining using the magnified intersections method (McGONIGLE et al., 1990).The abundance of AM fungus was also determined molecularly by analysing the expression levels of the *R. irregularis* elongation factor 1α *RiEF1α* and the expression levels of the mycorrhiza-induced phosphate transporter of *Z. mays ZmPht1;6* as an indicator of a functional AM symbiosis. Relative gene expression was calculated using the $2^{-\Delta CT}$ method with *EF1α* of the maize as internal control. Bars represent standard error. Different letters indicate significant differences ($P < 0.05$) among treatments.

Effect of Cu and mycorrhiza on the maize ionome

Nutrient content patterns of non-mycorrhizal and mycorrhizal plant tissues (shoot and root) were assessed by sparse partial least squares discriminant analysis (sPLSDA). This analysis revealed a strong impact of the Cu treatment on the shoot and root nutrient contents of both mycorrhizal and non-mycorrhizal plants. Interestingly, the mycorrhizal treatment affected the shoot ionome but not the root ionome. Although some differences were found between the shoot nutrient content profiles of mycorrhizal and non-mycorrhizal plants grown under control conditions, these differences were bigger in plants grown in soils supplemented with the highest Cu concentration (**Figure 2**).

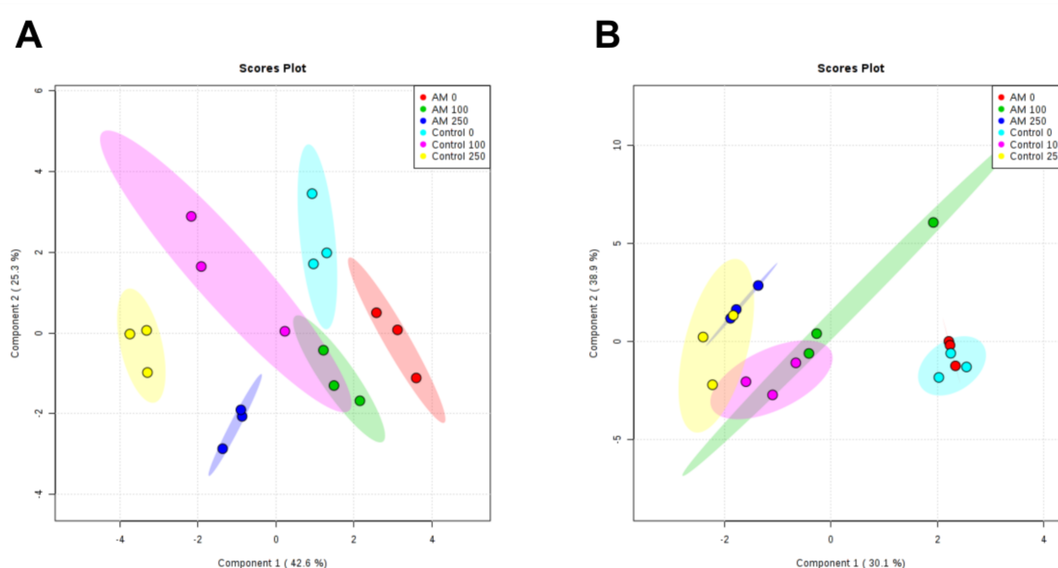

Figure 2. Effect of Cu toxicity and mycorrhizal inoculation on maize ionome. Shoot (A) and root (B) nutrients patterns were assessed by sPLSDA in non-mycorrhizal and mycorrhizal inoculated maize plants (AM) grown in Cu supplemented soils with 0, 100, 250 mg Cu soil^{-1}. Nutrient data were previously transformed with generalized logarithm transformation and scaled by the pareto method.

As expected, root and shoot Cu concentrations significantly increased with soil Cu content (**Table 2**). At 100 and 250 mg Kg^{-1} soil, shoot Cu concentration was significantly lower in mycorrhizal plants than in non-mycorrhizal plants. However, development of the symbiosis only decreased root Cu concentration when plants were grown in a soil supplemented with 100 mg Kg^{-1} soil (**Table 2**).

Table 2. Cu concentration in plant tissues subjected to the different Cu toxicity treatments. Cu concentration (ppm) in shoots and roots in non-mycorrhizal (NM) and mycorrhizal inoculated maize plants (MYC) grown in Cu supplemented soils with 0, 100, 250 mg Cu soil^{-1}. Values are means ± standard error. Different letters show statistically significant differences ($P < 0.05$) among treatments.

	0 (mg kg^{-1} soil)		**100 (mg kg^{-1} soil)**		**250 (mg kg^{-1} soil)**	
	NM	**MYC**	**NM**	**MYC**	**NM**	**MYC**
SHOOT	5.12 ± 0.37 **d**	4.71 ± 0.29 **d**	15.38±2.63 **b**	10.36±0.46 **c**	22.09±0.99 **a**	16.50±0.49 **b**
ROOT	22.37±0.95 **d**	21.76±0.89 **d**	754.60±43.44 **b**	436.63±79.74 **c**	1175.93±76.88**a**	1187.43±26.79**a**

Identification of **HMAs** *in* **Zea mays** *genome*

As a first step to analyse the role of metal efflux transporters on Cu detoxification in non-mycorrhizal and mycorrhizal plants, we searched for genes putatively encoding HMAs in maize. Our search identified a total of 12 maize genes putatively encoding heavy metal ATPases, which were named according to their closest HMA orthologs of *Arabidopsis thaliana* (**Table 3**). All of them were predicted to have the 8 transmembrane helices and all the domains typical of P_{-1B} type ATPases (Arguello, 2003; Arguello et al., 2007) (**Table 3**).

Table 3. Structural features of ZmHMA proteins. Their rice and *Arabidopsis* orthologs are indicated. Ch: Chromosome number; aa: amino acid; Sub. Localization: subcellular localization predicted with WoLF PSORT and TargetP algorithms; TM: putative transmembrane helices predicted with TOPCONS web server. References: **1.** Kim et al., 2009; **2.** Moreno et al., 2008; **3.** Seigneurin-Berny et al., 2006; **4.** Boutigny et al., 2014; **5.** Chaudhary et al., 2016; **6.** Satoh-Nagasawa et al., 2012; **7.** Takahashi et al., 2012; **8.** Yamaji et al., 2013; **9.** Chao et al., 2012; **10.** Gravot et al., 2004; **11.** Hussain et al., 2004; **12**. Liu et al., 2017; **13.** Morel et al., 2009; **14.** Park et al., 2012; **15.** Miyadate et al., 2011; **16.** Ueno et al., 2010; **17.** Che et al., 2019; **18.** Shao et al., 2018; **19.** Sasaki et al., 2014; **20.** Andres-Colas et al., 2006; **21.** Kobayashi et al., 2008; **22.** Huang et al., 2016; **23.** Deng et al., 2013; **24.** Abdel-Ghany et al., 2005; **25.** Shikanai et al., 2003; **26.** Catty et al., 2011; **27.** Sautron et al., 2016; **28.** Lee et al., 2012; **29.** Hirayama et al., 1999; **30.** Woeste and Kieber, 2000; **31.** Li and Lacey, 2017; **32.** Binder et al., 2010; **33.** Lee et al., 2007.

Protein name	Ch	aa	Sub. Localization	TM	At ortholog	Sub. Localization	Os ortholog	Sub. Localization
ZmHMA1	5	823	Chloroplast	8	AtHMA1 [1-4]	Chloroplast	OsHMA1 Uncharacterized	
ZmHMA2	5	1099	Plasma membrane	8	AtHMA2-4 [5]	AtHMA2 y 4: plasma membrane AtHMA3: tonoplast	OsHMA2 [6-8]	Plasma membrane
ZmHMA3.3	2	993	Tonoplast	8	AtHMA3 [5,9-14]	tonoplast	OsHMA3 [15-19]	Tonoplast
ZmHMA3.2	2	927	Tonoplast	8	AtHMA3	tonoplast	OsHMA3	
ZmHMA3.1	7	883	Tonoplast	8	AtHMA3	tonoplast	OsHMA3	
ZmHMA5.3	5	980	Tonoplast	8	AtHMA5 [20,21]	Plasma membrane	OsHMA4 [22]	Tonoplast
ZmHMA5.1	2	999	Plasma membrane	8	AtHMA5	Plasma membrane	OsHMA5 [23]	Plasma membrane
ZmHMA5.2	2	1036	Plasma membrane	8	AtHMA5	Plasma membrane	OsHMA5	Plasma membrane
ZmHMA6	4	928	Chloroplast	8	AtHMA6.PAA1 [4,24-28]	Chloroplast	OsHMA7 Uncharacterized	
ZmHMA7	4	998	Plasma membrane	8	AtHMA7.RAN 1 [29-32]	Post.Golgi	OsHMA6 Uncharacterized	
ZmHMA8	1	1003	Chloroplast	8	AtHMA8.PAA2 [24,27]	Chloroplast	OsHMA8 Uncharacterized	
ZmHMA9	9	1010	Plasma membrane	8	AtHMA7.RAN 1	Post.Golgi	OsHMA9 [33]	Plasma membrane

To explore the phylogenetic relationships of the maize HMA proteins with the HMA proteins from *A. thaliana* and *O. sativa*, an unrooted phylogenetic tree was constructed. The HMAs were divided into six different clusters. Each cluster groups genes from the three plant species, suggesting that the same cluster had a common ancestor. Based on sequence similarities and the relative positions in the tree, they were classified within the three groups of the HMA families: P1B-1 subgroup of Cu^+/Ag^+-ATPases (clusters III, IV, V and VI), P1B-2 subgroup of Zn/Cd/Pb-ATPases (cluster II) and P_{1B-4} subgroup of broad-specificity ATPases (cluster I) (**Figure 3**).

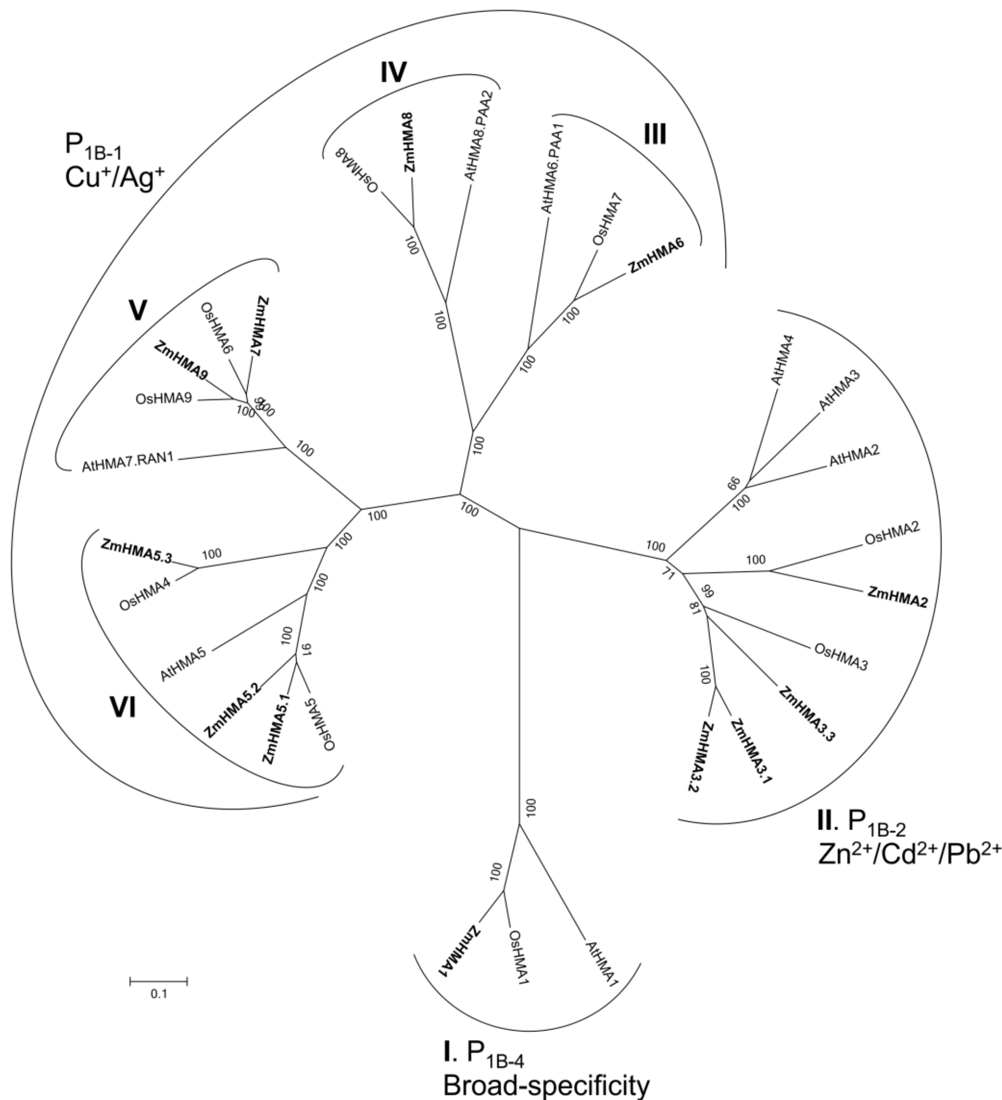

Figure 3. Phylogenetic relationships among maize, rice and *Arabidopsis* HMA proteins. The unrooted Neighbor-joining tree was generated using MEGA v. 6 with 1000 bootstrap replicates. *Zea mays* HMA proteins are in bold. Organisms: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Zm, *Z. mays.* Bootstrap values above 70 and supporting a node used to define a cluster are indicated.

In silico predictions of subcellular localization using TargetP and WoLF PSORT suggest that the different maize HMAs are targeted to different cellular membranes (**Table 3**). ZmHMA1, ZmHMA6 and ZmHMA8 contain a chloroplast transit peptide and, as their *Arabidopsis* and rice

orthologs, localize with the highest probability to the chloroplasts. ZmHMA5.2 and ZmHMA5.3 also contain the chloroplast targeting peptide but based on the WoLF PSORT predictions and on their phylogenetic relationships with the *Arabidopsis* and rice proteins, they are predicted to localize with much higher probability to the plasma membrane and tonoplast, respectively. ZmHMA5.1, ZmHMA2, ZmHMA7 and ZmHMA9 were strongly predicted to localize in the plasma membrane. However, ZmHMA3.1, HMA3.2 and ZmHMA3.3 were predicted with almost the same probability to be targeted to different intracellular membranes, including the endoplasmic reticulum and tonoplast. These predictions agree with the phylogenetic analysis and suggest that they localize to the intracellular membranes, most likely to the tonoplast, as their *Arabidopsis* orthologs.

ZmHMAs *are differentially regulated in roots and shoots*

Transcript levels of *ZmHMA1, ZmHMA3.2, ZmHMA5.1, ZmHMA6* and *ZmHMA8* were significantly higher in shoots than in roots; whereas transcript levels of *ZmHMA5.2* and *ZmHMA9* were higher in roots. Non-significant differences in transcript levels of *ZmHMA2*, *ZmHMA3.3, ZmHMA5.3* and *ZmHMA7* were found between roots and shoots. *ZmHMA3.1* expression was not detected in any of the tissues (**Figure 4**).

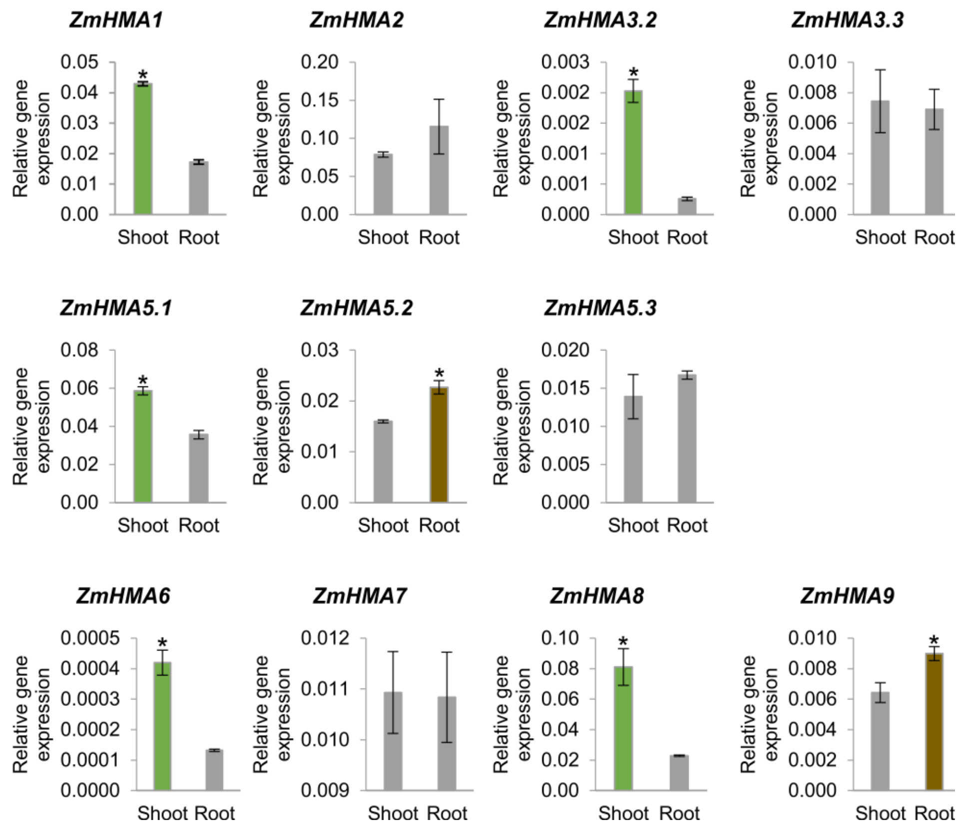

Figure 4. *ZmHMAs* expression levels in different plant tissues (shoot and root). Relative gene expression levels were calculated by the $2^{-\Delta CT}$ method using the elongation factor 1α of *Z. mays* (*ZmEF1α*) as a normalizer. Bars represent standard error. Asterisks show statistically significant differences ($P < 0.05$) between shoot and root.

Effect of Cu availability and mycorrhiza on **ZmHMAs** *gene expression*

Transcripts of *ZmHMA3.1* were not detected in any of the conditions tested in our study.In non-mycorrhizal plants, shoot *ZmHMA*s expression was not affected by Cu toxicity (**Figure 6**). Development of the symbiosis in the non-contaminated soil neither regulated ZmHMAs expression. Relative to the mycorrhizal plants grown in the non-contaminated soil, ZmHMA gene expression in shoots of mycorrhizal plants did not follow a general pattern. *ZmHMA5.3* expression increased only in shoots of mycorrhizal plants grown in the soil supplemented with 100 mg Kg^{-1} soil and a slight increase in ZmHMA3.3 was observed at the highest Cu soil concentration (**Figure 5**).

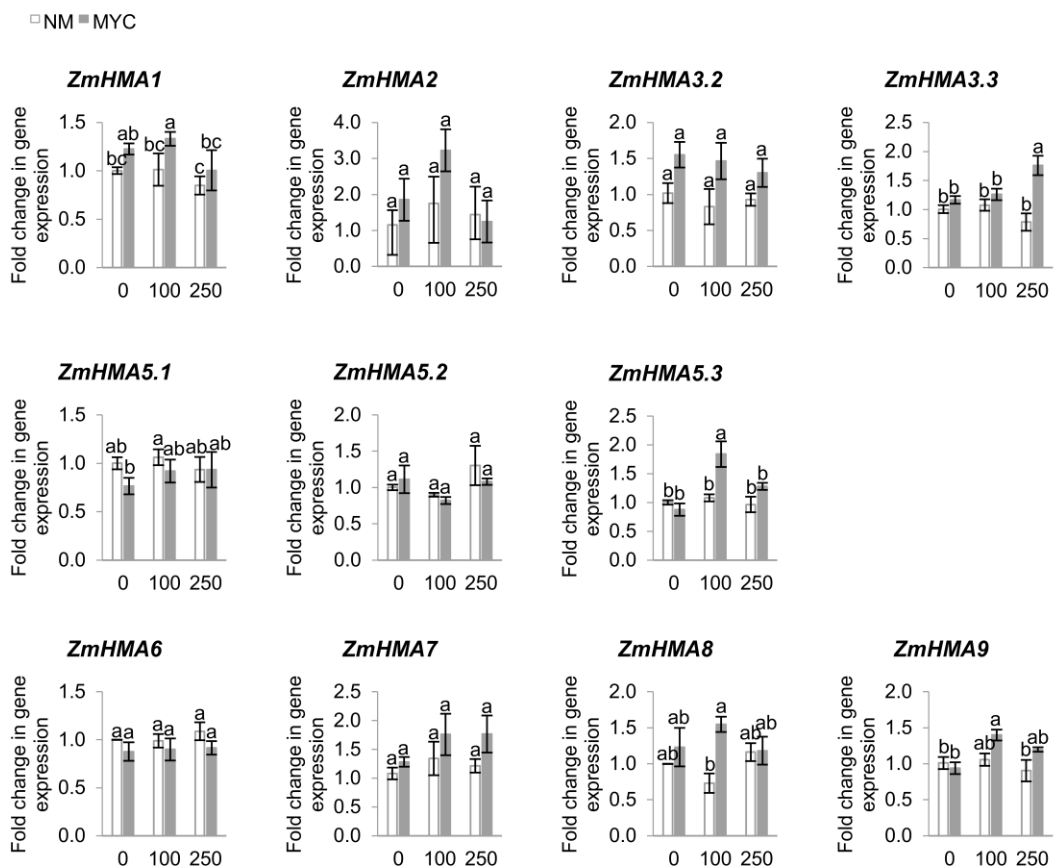

Figure 5. Effect of Cu toxicity and mycorrhiza inoculation on shoot *ZmHMA* gene expression. Relative gene expression *was* calculated by the $2^{-\Delta\Delta CT}$ method using the elongation factor 1α of *Z. mays* (*ZmEF1α*) as a normalizer. Bars represent standard error; * $P < 0.05$ statistically significant in comparison to the corresponding control value.

In non-mycorrhizal roots, *ZmHMA5.1* and *ZmHMA 5.2*, whose gene products were predicted to localize to the plasma membrane, are up-regulated by Cu toxicity. While *ZmHMA5.1* expression increased in roots of plants grown in soils contaminated with 100 and 250 mg Kg^{-1} soil, *ZmHMA5.2* expression only increased at the highest Cu concentration. In contrast, Cu

toxicity decreased *ZmHMA8* and *ZmHMA9* expression in non-mycorrhizal roots, being *ZmHMA8* down-regulated only at the highest Cu concentration. Interestingly, development of the symbiosis in the non-contaminated soil up-regulates transcription of *ZmHMA3.2* but down-regulates *ZmHMA8* and *ZmHMA9* gene expression. Up-regulation of *ZmHMA3.2*, whose gene product was predicted to localize to the tonoplast, was also observed in mycorrhizal roots of plants grown in the contaminated soils. As observed in the non-mycorrhizal roots, Cu toxicity up-regulates *ZmHMA5.1* and *ZmHMA5.2* expression in mycorrhizal roots, although *ZmHMA5.1* only at the highest Cu concentration and *ZmHMA5.2* to a lower extent. In mycorrhizal roots, Cu toxicity increases the expression of two additional *ZmHMA* genes, *ZmHMA3.3* and *ZmHMA5.3* (**Figure 6**).

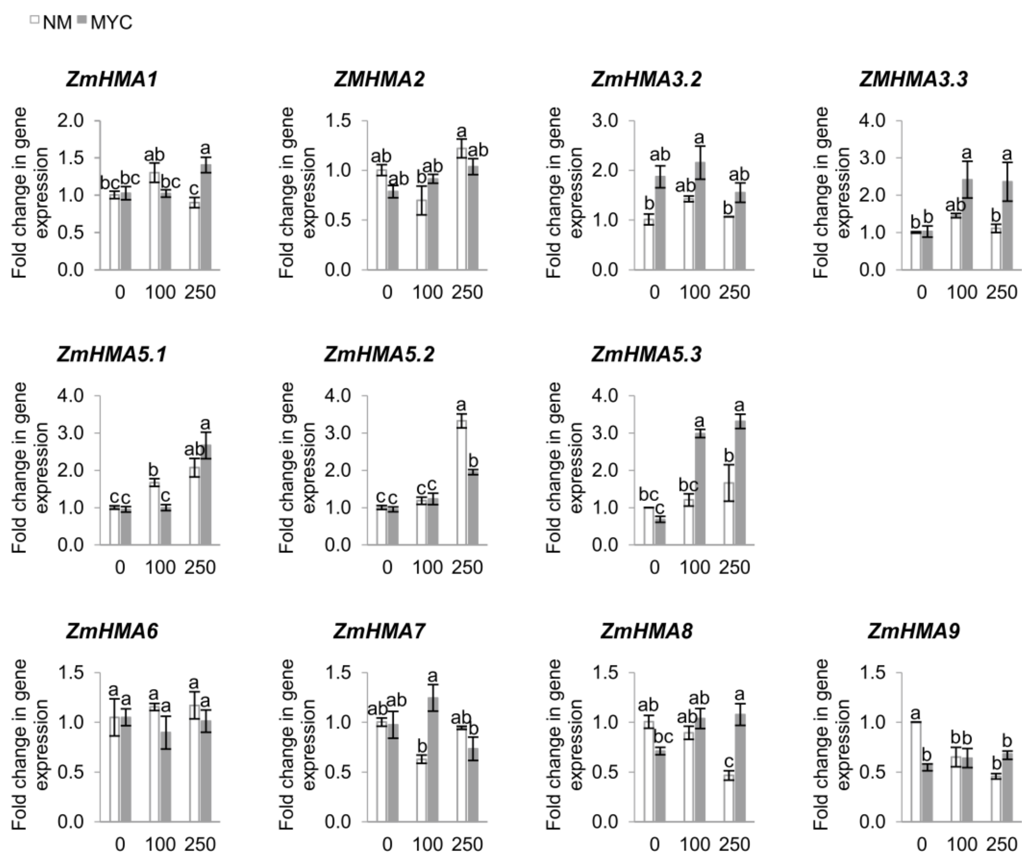

Figure 6. Effect of Cu toxicity and mycorrhiza inoculation on root *ZmHMA* gene expression. Relative gene expression *was* calculated by the $2^{-\Delta\Delta CT}$ method using the elongation factor 1α of *Z. mays* (*ZmEF1α*) as a normalizer. Bars represent standard error; * P < 0.05 statistically significant in comparison to the corresponding control value

Discussion

Different mechanisms have been proposed to explain the protective effect of AM fungi to Cu toxicity. The finding that growth of the mycorrhizal plants was less affected by Cu toxicity than that on non-mycorrhizal plants agrees with previous observations in maize and other plant

species that AM mitigate metal-induced stress in the host plant (Cicatelli et al., 2010; Pallara et al., 2013; Merlos et al., 2016). It is well known that AM increases host nutrient acquisition, especially phosphorous (Ferrol et al., 2018; Wipf et al., 2019). However, our ionomic analyses revealed that inoculation with *R. irregularis* did not significantly increased plant nutrient status, as mycorrhizal and non-mycorrhizal plants had similar or lower nutrient concentrations, which might be due to the known dilution effect produced as a consequence of the higher biomass of the mycorrhizal plants. Therefore, the lower Cu content of the mycorrhizal plants could be also partially due to this dilution effect.

Our results indicate that the *Z. mays* genome contains 12 genes putatively encoding HMAs. The lengths of these sequences significantly varied, implying a high degree of complexity among the HMA genes. All the encoded proteins have the characteristic structural features of this P_{1B}-type ATPase subfamily, including 8 transmembrane domains. The plant HMA family is divided in three groups, according to their structures and functions (Arguello, 2003; Arguello et al., 2007). P_{1B-1} subgroup is composed of highly specific Cu^{+}/Ag^{+} transporting ATPases, P_{1B-2} subgoup clusters $Zn^{2+}/Cd^{2+}/Pb^{2+}$ ATPases and P_{1B-4} subgroup is composed of a broad metal-specificity ATPases including Cu-ATPases. In this study, ZmHMA5.1, ZmHMA5.2, ZmHMA5.3, ZmHMA6, ZmHMA7, ZmHMA8 and ZmHMA9 have been included in the Cu^{+}/Ag^{+} group, ZmHMA1 in the P_{1B-4} subgroup and ZmHMA2, ZmHMA3.1, ZmHMA3.2 and ZmHMA3.3 in the P_{1B-2} subgroup of $Zn^{2+}/Cd^{2+}/Pb^{2+}$ATPases.

Regarding the P_{1B-1} subgroup, up-regulation by Cu toxicity of *ZmHMA5.1* and *ZmHMA5.2* in roots of non-mycorrhizal and mycorrhizal plants and of *ZmHMA5.3* in mycorrhizal roots suggests a role for these ATPases in Cu detoxification. Given that the proteins encoded by *ZmHMA5.1* and *ZmHMA5.2* were predicted to be localized at the plasma membrane, they should act as efflux Cu-pumps, as it was suggested for other HMA5-like proteins. For instance, the *A. thaliana* AtHMA5 interacts with ATX1-like Cu chaperones, proteins with a predominant function in Cu to P-type ATPases, and functions in Cu detoxification of roots (Andres-Colas et al., 2006; Kobayashi et al., 2008). A similar role has been attributed to SvHMA5II of the metallophyte plant *Silene vulgaris*, a reticulum endoplasmic located transporter that moves to the plasma membrane under Cu exposure leading to Cu efflux in roots (Li et al., 2017). Since in non-mycorrhizal roots *ZmHMA5.1* and *ZmHMA5.2* transcripts accumulate in a Cu-dependent manner, the finding that in mycorrhizal roots *ZmHMA5.1* was only up-regulated at the highest Cu concentration and that *ZmHMA5.2* expression was more highly induced in non-mycorrhizal roots suggests that Cu cytosolic levels are lower in the cells of the mycorrhizal roots. This hypothesis is supported by the observed preferential accumulation of metals in the intraradical fungal structures than in root cells of a mycorrhizal root and in the external mycelium (Turnau et al., 1993; Wu et al., 2016).

Up-regulation of two plasma membrane Cu efflux proteins by Cu suggests that there might exist some functional redundancy between these two proteins.

On the other hand, *ZmHMA5.3*, whose gene expression is up-regulated by Cu toxicity only in mycorrhizal tissues, is phylogenetically closely related to the tonoplast Cu transporter OsHMA4 of *O. sativa*, a protein that transports Cu ions from the cytosol to the vacuoles preventing its toxicity (Huang et al., 2016). In contrast with what happens with *OsHMA4* and the two vacuolar HMAs of *Cucumber sativus CsHMA5.1* and *CsHMA5.2*, which are mainly expressed in roots and contribute to metal detoxification in the root vacuoles reducing, therefore, root to shoot Cu translocation (Migocka et al., 2015; Huang et al., 2016). *ZmHMA5.3* displayed similar expression levels in roots and shoots. This expression pattern was also observed for the vacuolar *SvHMA5I* of *S. vulgaris* (Li et al., 2017) and, as it has been proposed by these authors, suggests that ZmHMA5.3 plays a dual role in shoot Cu protection, an indirect role by preventing root to shoot Cu translocation through its accumulation in the root vacuole and direct role through vacuolar compartmentalization of the Cu that reaches the shoot. The finding that Cu toxicity up-regulates *ZmHMA5.3* expression only in roots and shoots of mycorrhizal plants suggests that development of the symbiosis under Cu toxic conditions regulates plant Cu homeostasis and specifically induces the expression of certain proteins involved of Cu detoxification potentiating in this way plant Cu tolerance. This hypothesis agrees with previous observations in different plant species that AM fungi induce up-regulation of plant genes encoding proteins involved in metal detoxification, such as phytochelatin synthases and metallothioneins (Cicatelli et al., 2010; Pallara et al., 2013) and with an increased accumulation of phytochelatins (Merlos et al., 2016). The finding that Cu toxicity increased *ZmHMA5.3* expression in shoots of mycorrhizal plants indicates that development of the symbiosis induces a systemic effect on the plant detoxification mechanism.

ZmHMA1, ZmHMA6 and *ZmHMA8* whose encoded proteins were predicted to have chloroplast signal peptide, were highly expressed in shoots of all treatments. However, they were also detected in roots, which suggests a role for these proteins in both green and non-green plastids. Their *A. thaliana* orthologs are required for Cu delivery into the chloroplast. AtHMA6 (also known as PAA1) and AtHMA8 (also known as PAA2) are proposed to function sequentially in Cu transport over the plastid envelope and the thylakoid membrane, respectively, being crucial for Cu delivery to Cu containing proteins in the chloroplast, such as the stromal Cu/Zn superoxide dismutase 2 (CSD2) and plastocyanin (Shikanai et al., 2003; Abdel-Ghany et al., 2005; Cohu and Pilon, 2007). AtHMA1 is a Ca^{2+}/ heavy metal ATPase involved in the transport of multiple cations between the chloroplast and the cytosol (Moreno et al., 2008; Kim et al., 2009; Boutigny et al., 2014). Interestingly, while Cu toxicity did not affect shoot expression levels of any of the

chloroplast tagged HMAs, *ZmHMA8* expression was down-regulated by Cu toxicity in roots of non-mycorrhizal plants may be to preserve root plastid function under these conditions.

On the other hand, ZmHMA7 and 9 are phylogenetically close to OsHMA6 and 9, respectively, and clusters in the same subgroup than AtHMA7/RAN1. *ZmHMA9* was highly expressed in roots while no significance differences in *ZmHMA7* expression between shoots and roots were detected. AtHMA7/RAN1 participates in the delivery of Cu to ethylene receptors which need it as a cofactor and by homology to *Saccharomyces cerevisiae* ScCCC2 is proposed to be localized in the post-Golgi compartment (Hirayama et al., 1999; Rodriguez et al., 1999; Woeste and Kieber, 2000; Keunen et al., 2016). In rice, only OsHMA9 has been characterized in detail, a plasma membrane efflux protein which has been related with Cu and Zn detoxification, while its role in ethylene signalling remains to be elucidated (Lee et al., 2007). A plasma membrane localization was also predicted for ZmHMA7 and 9, although gene expression of both were down-regulated in roots under toxic Cu conditions and nearly unaffected in shoots by the Cu treatment, suggesting that their role is probably not linked with Cu detoxification.

In addition to these well reported Cu-ATPases, ZmHMA3.3 included in the P_{IB-2} subgroup, are probably involved in Cu homeostasis. Remarkably, *ZmHMA3.3* was highly up-regulated by Cu toxicity in roots and shoots of mycorrhizal plants, similarly to *ZmHMA5.3* gene expression pattern. As proposed before for ZmHMA5.3 and its closest ortholog in rice OsHMA3, ZmHMA3.3 is probably tagged to the tonoplast, preventing Cu toxicity by accumulation in root and shoot vacuoles. However, HMA3-like proteins have been mostly related with Zn2+ and/or Cd2+transport, including rice OsHMA3 (Gravot et al., 2004; Hussain et al., 2004; Morel et al., 2009; Wong and Cobbett, 2009; Ueno et al., 2010; Miyadate et al., 2011; Park et al., 2012). Furthermore, HMA3-like proteins have been identify as a key players on the metal hyperaccumulating capability of some plant species such as *TcHMA3* of the Cd-hyperaccumulating "Ganges" ecotype of *Thlaspi caerulescens* and *SpHMA3* of the Cd/Zn hyperaccumulator *Sedum plumbizincicola* which are mainly expressed in shoots allowing them to accumulate high levels of these metals in aboveground tissues without suffering damage (Ueno et al., 2011; Liu et al., 2017). Thus, the capability of ZmHMA3.3 to transport other metals should not be discarded. Finally, ZmHMA3.2 was predicted to be tagged with almost the same probability to different intracellular membranes including endoplasmic reticulum and tonoplast. Interestingly, gene expression of this transporter was up-regulated in roots from mycorrhizal inoculated plants regardless of the Cu treatment. Further studies are required to understand ZmHMA3.2 function.

Until the date Only up-regulation of *GmHMA19*, a HMA1-like P_{IB-4} ATPase, by AM in shoots under Cadmium toxicity have been recently reported in soybean (Cui et al., 2019).

In summary, *Z. mays* genome contains 12 genes putatively encoding HMAs. As expected for HMAs belonging to P_{1B-2} subgroup, ZmHMA2, ZmHMA3.1 and ZmHMA3.2 were not regulated by Cu. ZmHMA1, ZmHMA6 and ZmHMA8 were predicted to be tagged at the chloroplast as their AtHMA orthologs and preferentially expressed in areal tissues although non-significant changes in their shoot gene expression patterns were detected by Cu toxicity. Our results strongly suggest a role in Cu detoxification of ZmHMA5.1 and 5.2 probably acting as a Cu efflux plasma membrane pumps and ZmHMA5.3 and ZmHMA3.3 probably sequestering Cu ions in shoot and root vacuoles reducing Cu translocation to aerial tissues. Interestingly, enhanced expression of these two putative tonoplast transporters were found in mycorrhizal plants grown under Cu toxicity. Finally, ZmHMA7 and ZmHMA9 were predicted to be located at the plasma membrane and they were down-regulated by Cu toxicity, their impact on ethylene signaling should be tested as this is the main function of their closely related AtHMA7/RAN1 transporter. Although this work provides some insights into ZmHMAs identification and function, more detailed studies of characterization are required to clarify their role. On the other hand, the presence or not of mycorrhiza had also a great impact on gene expression of *ZmHMA* transporters underscoring the importance of AM symbiosis in plant Cu distribution and nutrition.

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Discusión General

DISCUSIÓN GENERAL

Con el fin de obtener una visión global de la contribución de esta tesis doctoral al conocimiento de la homeostasis de Cu en la simbiosis MA, a continuación, se presentan y discuten de manera conjunta los resultados más relevantes obtenidos en los diferentes capítulos.

El cobre, al igual que otros metales de transición como el Zn y el Fe, son requeridos en bajas concentraciones como micronutrientes pero cuando están en exceso resultan tóxicos. El Cu actúa como cofactor de numerosas proteínas implicadas en procesos esenciales para la vida, tales como la respiración y la fotosíntesis. Sin embargo, en exceso resulta tóxico debido a su capacidad de desplazar a otros iones metálicos de centros catalíticos o motivos estructurales presentes en cuproproteínas y a través de la generación de especies de oxígeno reactivas que pueden causar daño oxidativo en diferentes moléculas biológicas. Con el fin de mantener los niveles de Cu en niveles fisiológicos todos los organismos han desarrollado complejas redes homeostáticas, en la que proteínas transportadoras de membrana que controlan el influjo y eflujo del metal resultan esenciales para mantener unas concentraciones de Cu equilibradas, tal y como hemos visto reflejado en los resultados obtenidos en los diferentes capítulos de esta tesis doctoral. Esto es de especial relevancia en los organismos que se encuentran en íntimo contacto con el suelo, como las plantas y los hongos MA, ya que están expuestos a concentraciones de Cu ambientales variables. Aunque el Cu suele estar presente en los suelos como elemento traza, las actividades antropogénicas han provocado la contaminación de los suelos con este metal. Numerosos estudios han puesto de manifiesto la capacidad de las MA de beneficiar el desarrollo de las plantas tanto en suelos pobres en Cu como en suelos contaminados por este metal. Se han descrito diferentes mecanismos para explicar el efecto bioprotector de las micorrizas en las plantas frente a estrés por metales pesados. Sin embargo, los sistemas de transporte de Cu en la simbiosis son desconocidos. En esta tesis doctoral hemos podido identificar algunas de las proteínas transportadoras que podrían jugar un papel relevante en la homeostasis de Cu de ambos simbiontes, especialmente en el simbionte fúngico.

El hongo micorrícico arbuscular expresa dos genes codificantes de transportadores de Cu tipo CTR, RiCTR1, RiCTR2 y dos variantes de empalme alternativo de un tercer gen *RiCTR3* (*RiCTR3A* y *RiCTR3B*). *RiCTR1* se expresa mayoritariamente en el micelio extrarradical y codifica un transportador de membrana implicado en la captación de Cu del medio. *RiCTR2* se expresa predominantemente en el micelio intrarradical y sería el responsable de la movilización de las reservas vacuolares de Cu. Los transportadores RiCTR1 y 2 son semejantes a los descritos anteriormente para otros hongos, así como en la levadura *S. cerevisiae*, siendo RiCTR3 el miembro más diferente. En la levadura modelo *S.cerevisiae* los transportadores de membrana ScCTR1 y ScCTR3 (Peña et al., 2000) son redundantes funcionalmente estando ambos implicados en la captación de Cu, mientras que ScCTR2 (Rees et al., 2004) es el encargado de

movilizar las reservas vacuolares. El tercer gen *RiCTR3* produce dos variantes de empalme alternativo, *RiCTR3A* y *RiCTR3B*, que se expresan mayoritariamente en el ERM. La activación transcripcional de *RiCTR3A* por toxicidad de Cu y la capacidad de su producto génico de revertir la sensibilidad al Cu del mutante *Δyap-1* de levadura sugiere que RiCTR3A podría funcionar como un receptor de Cu necesario para la activación de los mecanismos involucrados en la tolerancia al Cu.

El genoma de *R. irregulairs* tiene cuatro genes que codifican potencialmente P1b-ATPasas, también conocidas como ATPasas de metales pesados (HMAs). Estos transportadores acoplan la hidrólisis de ATP al transporte de metales pesados a través de diferentes membranas celulares. La presencia de tres ATPasas tipo CCC2 en *R. irregulairs* representa una expansión respecto a las presentes en otros hongos de referencia. *RiCCC2.1-3* son ortólogos a *CCC2* de *Saccharomyces cerevisaie* que codifica una Cu-ATPasa localizada en el aparato de Golgi que transfiere Cu a cuproproteinas. Sin embargo, el hecho de que *R. irregularis* tenga varias isoformas CCC2.1, podría implicar una diferente localización, posiblemente vacuolar como ocurre con algunas ATPasas de plantas implicadas en la transferencia de metales desde el citosol al interior de las vacuolas, tales como los transportadores OsHMA3 y 4 de *Oryza sativa* y AtHMA3 de *Arabidopsis thaliana.* Sin embargo, se requieren estudios de caracterización funcional y de localización de las diferentes RiCCC2 identificadas, para apoyar esta hipótesis. Por otro lado, *RiCRD1* es ortólogo de *CaCRD1* de la levadura patógena *Candida albicans*, que codifica una P_{1B}-ATPasa implicada en el eflujo del exceso de Cu fuera de la célula, proporcionándole resistencia a este metal. La fuerte inducción de *RiCRD* en los ERM expuestos a diferentes tratamientos de toxicidad de Cu sugiere que el eflujo del exceso del metal fuera del citosol es uno de los principales determinantes de la resistencia a Cu en *R. irregularis*. Esto es de especial relevancia en hongos MA, ya que la única estrategia de tolerancia a metales descrita basada en el eflujo de metales en hongos MA es un transportador de arsenico RiArsA que actúa en coordinación con el transportador RiPT implicado en la captación de fósforo (Gonzalez-Chavez Mdel et al., 2011). Adicionalmente la observación de algunas esporas azules en los ERM expuesto a concentraciones tóxicas de Cu es consistente con estudios previos de Cornejo y colaboradores (2013) y es indicativa de que el hongo compartimentaliza parte del Cu en algunas esporas de la colonia fúngica, Por otro lado, la demostración mediante técnicas de hibridación *in situ* de la presencia de transcritos de *RiCRD* en los arbúsculos desarrollados en células corticales de raíces micorrizadas de tomate, sugiere que éste podría ser el transportador implicado en la transferencia del Cu del hongo al espacio periarbuscular. La predicción de la localización de este transportador en la membrana plasmática también avala ambas hipótesis. Así RiCRD podría desempeñar un papel dual en la detoxificación de Cu en el ERM y en la nutrición simbiótica de Cu en el IRM, siendo

un transportador clave en la homeostasis de Cu de *R. irregularis* y en general, en la simbiosis MA.

Teniendo en cuenta todos estos resultados de manera conjunta proponemos el siguiente modelo para el transporte y distribución de Cu en una raíz micorrizada por el hongo MA *R. irregularis.* La captación del Cu del medio tiene lugar en el extenso entramado de hifas que el hongo desarrolla en el suelo probablemente mediada por la acción del transportador de membrana RiCTR1, cuya expresión aumenta en condiciones de deficiencia y se reprime bajo condiciones de toxicidad. Una vez en el citosol debido a la alta reactividad del Cu, debería ser quelado por la acción de chaperonas. Por otro lado, tal y como se ha comentado anteriormente, alguna de ATPasas de metales pesados tipo CCC2, como ocurre en algunas ATPasas de plantas (Williams and Mills, 2005), podría localizarse en la vacuola contribuyendo a la acumulación de Cu en este orgánulo. Otros miembros de esta familia es probable que se encuentren en el aparato de Golgi como la ATPasa de *S. cerevisiae* CCC2 (Yuan et al., 1997) desde donde transportarían Cu a cuproproteínas. El cobre debe ser translocado desde el micelio extrarradical hasta el micelio intrarradical, para finalmente alcanzar los arbúsculos, probablemente sea transportado en las vacuolas ya que son los principales sitios de acumulación y movilización de metales pesados. La co-localización del Cu y otros metales junto con los polímeros de poliP en las vacuolas apoya esta idea, además los metales podrían ser estabilizados por las cadenas de poliP, tal y como plantearon González-Guerrero y colaboradores en el 2008. Una vez en los arbúsculos, el Cu debe ser liberado primero de las vacuolas, probablemente mediante la acción de RiCTR2 ,que se expresa mayoritariamente en el micelio intrarradical y está implicado en la movilización del Cu vacuolar. Posteriormente sería exportado al espacio periarbuscular probablemente a través del transportador de membrana plasmática RiCRD, para finalmente ser adquirido por la planta hospedadora a través de proteínas transportadoras presentes en la membrana periarbuscular. En este sentido, Gómez y colaboradores (2009) y datos aún no publicados de un miembro de nuestro equipo de investigación identificaron un transportador perteneciente a la familia de transportadores de Cu CTR/COPT en *Medicago truncatula* y *Zea mays*, respectivamente, que se inducía fuertemente por micorrización y que podrían estar implicados en la captación de Cu del espacio periarbuscular.

Bajo condiciones de deficiencia de Cu la inducción de *RiCTR1* y la represión de *RiCRD* en la fase *in planta* del hongo, refleja el requerimiento de Cu por parte del simbionte fúngico que podría competir por el Cu con la planta hospedadora. La disminución de la colonización arbuscular bajo condiciones limitantes de Cu también refleja la importancia de este micronutriente en el desarrollo de la simbiosis.

Por otro lado, si el Cu alcanzase niveles tóxicos en el medio, sería probablemente detectado por RiCTR3, presente en la membrana plasmática y cuya expresión génica se induce

fuertemente en el ERM bajo condiciones de toxicidad de Cu. Esta proteína, sería la encargada sensar el exceso de Cu en el medio desencadenando la activación de los mecanismos de tolerancia frente a este metal. Además, se induciría RiCRD que actuaría como bomba de eflujo de Cu y, en menor medida, la metalotioneina RiMT y las fitoquelatinas contribuirían a acomplejar el Cu que lograse alcanzar el citosol.

La importancia de la simbiosis micorrícico arbuscular en la regulación de la expresión de transportadores a nivel de la planta bajo condiciones de toxicidad de Cu también ha quedado reflejada en esta Tesis Doctoral. La inoculación de un cultivar sensible al Cu de *Zea mays* con el hongo MA *R. irregularis* modificó la respuesta fisiológica de la planta a la toxicidad de Cu y la expresión génica de HMAs. Las plantas micorrizadas presentaron una mayor biomasa que las no micorrizadas y una concentración menor de Cu en sus tejidos. Curiosamente, aunque se encontraron algunas diferencias en el contenido de nutrientes entre la parte aérea de plantas micorrizadas y no micorrizas cultivadas bajo condiciones control, estas diferencias fueron mayores en las plantas cultivadas en suelos contaminados con la concentración más alta de Cu. De los 12 genes que codifican potencialmente HMAs en plantas de maiz, cuatro (*ZmHMA3.3, ZmHMA5.1, ZmHMA5.2* y *ZmHMA5.3*) se activan transcripcionalmente por toxicidad de Cu. Curiosamente, la inducción en la expresión de *ZmHMA3.3* y *ZmHMA5.3* solamente tuvo lugar en la parte aérea y raíz de las plantas micorrizadas. Estos transportadores probablemente se encuentren en las vacuolas, y como sus ortólogos en arroz, promuevan la acumulación de metales pesados bajo condiciones de toxicidad (Ueno et al., 2010; Miyadate et al., 2011; Huang and Deng, 2016). Nuestros resultados sugieren que ZmHMA3.3 y ZmHMA5.3 podrían desempeñar un papel dual en la protección frente a la toxicidad de Cu de la parte aérea de las plantas micorrizadas disminuyendo la translocación de este metal de la parte aérea a la raíz mediante su acumulación en las vacuolas de las raíces y mediante la compartimentilización vacuolar de Cu que alcanzase la raíz. Estos datos sugieren que el desarrollo de la simbiosis bajo condiciones de toxicidad de Cu puede regular los mecanismos de homeostasis de Cu en la planta hospedadora, a través de la inducción específica de ciertas proteínas involucradas en la detoxificación de Cu, potenciando así la tolerancia a Cu de la planta. Estos resultados concuerdan con otros estudios en los que en diferentes plantas hospedadoras también se ha encontrado un efecto protector de las micorrizas frente a condiciones tóxicas de Cu, así como la inducción de sistemas antioxidantes o que contribuyen a la quelación del metal en el citosol como metalotioneínas y fitoquelatinas (Cicatelli et al., 2010; Pallara et al., 2013; Merlos et al., 2016), sin embargo, hasta la fecha no se había descrito ninguna estrategia basada la regulación diferencial de transportadores implicados en el transporte de Cu. Solamente se ha descrito una P_{1B}-ATPasa tipo HMA1, posiblemente cloroplástica, cuya inducción se ha relacionado recientemente con una mayor tolerancia a Cd en plantas micorrizadas de soja (Cui et al., 2019).

Finalmente, cabe destacar la importancia del Cu en la simbiosis MA que ha quedado reflejada en todos los capítulos ya que tanto la deficiencia de cobre como la toxicidad han provocado una menor micorrización de las plantas. Aunque esta Tesis supone un gran avance en el conocimiento de la homeostasis de Cu en la simbiosis MA determinada por los sistemas de transporte, se requieren más estudios para entender completamente el funcionamiento de esta compleja red de transportadores y como su regulación diferencial puede influir en el estatus nutricional o tolerancia de ambos simbiontes, y por ende en el desarrollo de la simbiosis MA.

Conclusiones

CONCLUSIONES

1. El genoma del hongo micorrícico arbuscular *Rhizophagus irregularis* tiene siete marcos abiertos de lectura que codifican potencialmente transportadores de cobre pertenecientes a las familias de transportadores de cobre (CTR) y P_{1B}-ATPasas, también denominadas ATPasas de metales pesados (HMA).
2. La familia CTR de *Rhizophagus irregularis* está formada por dos miembros: RiCTR1 y RiCTR2. *RiCTR1* se expresa mayoritariamente en el micelio extrarradical y codifica un transportador de membrana responsable de la captación de Cu del medio. *RiCTR2* se expresa mayoritariamente en el micelio intrarradical y codifica un transportador de Cu implicado en la movilización de las reservas vacuolares de Cu bajo condiciones de deficiencia en este metal.
3. El gen *RiCTR3* produce mediante empalme alternativo dos transcritos: *RiCTR3A y RiCTR3B. RiCTR3A* codifica una proteína de membrana de plasmática que revierte la sensibilidad al Cu del mutante de levadura *Δyap* y que se activa transcripcionalmente bajo condiciones de toxicidad de Cu, por lo que podría tener un papel relevante como receptor de Cu necesario para la activación de los mecanismos de tolerancia al Cu.
4. El genoma de *Rhizophagus irregularis* tiene tres genes que codifican potencialmente Cu-ATPasas del tipo CCC2 de *Saccharomyces cerevisiae*, que se localiza en el aparato de Golgi y le transfiere Cu a cuproproteínas, lo que supone una expansión respecto a las presentes en otros hongos.
5. *RiCRD* potencialmente codifica una ATPasa de metales que membrana plasmática que se expresa en los arbúsculos y que se activa transcripcionalmente bajo condiciones tóxicas de Cu en el micelio extrarradical, lo que indica que RiCRD desempeña un papel dual en la detoxificación del exceso de Cu y en la transferencia del Cu desde el simbionte fúngico al espacio perarbuscular.
6. La reducción en la colonización arbuscular bajo condiciones limitantes de Cu refleja la importancia del Cu como micronutriente esencial en el desarrollo de la simbiosis.
7. El genoma de *Zea mays* tiene doce secuencias que codifican potencialmente ATPasas de metales pesados, de las cuales cuatro (*ZmHMA3.3, ZmHMA5.1, ZmHMA5.2* y *ZmHMA5.3*) se inducen bajo condiciones de toxicidad de Cu.
8. La mayor tolerancia de las plantas micorrizadas de maíz al exceso de Cu se debe en parte a la inducción específica de dos posibles Cu-ATPasas vacuolares, *ZmHMA3.3 y ZmHMA5.3* en la parte aérea y raíz de las plantas micorrizadas. Estos transportadores podrían desempeñar un papel dual en la protección de la parte aérea frente al Cu, disminuyendo la translocación de Cu a la parte aérea mediante su acumulación en las vacuolas de las raíces y mediante la compartimentalización vacuolar de Cu que logra alcanzar la parte aérea.

Conclusions

CONCLUSIONS

1. The arbuscular mycorrhizal fungus *Rhizophagus irregularis* contains in its genome seven open reading frames, which potentially encode copper transporters belonging to the CTR family of copper transporters and to the P_{IB}-ATPase subfamily, also known as Heavy Metal ATPases (HMA).
2. The *Rhizophagus irregularis* CTR family is composed by two members: RiCTR1 and RiCTR2. *RiCTR1* is more highly expressed in the extraradical mycelia and encodes a plasma membrane Cu transporter that mediates Cu acquisition from the environment. *RiCTR2* is more highly expressed in the intraradical mycelia and encodes a vacuolar Cu transporter that is involved in Cu mobilization of vacuolar stores under Cu-limiting conditions.
3. *RiCTR3* produces, as a consequence of an alternative splicing event, two transcripts: *RiCTR3A* and *RiCTR3B*. *RiCTR3A* is strongly up-regulated by Cu toxicity and encodes a plasma membrane protein that rescues Cu sensitivity of the *yap1Δ* mutant yeast. RiCTR3A might function as a Cu receptor that activates downstream signal transduction pathways involved in Cu tolerance.
4. The *Rhizophagus irregularis* genome harbors three genes, *RiCCC2.1*, *RiCCC2.2* and *RiCCC2.3*, whose gene products are orthologs of the *Saccharomyces cerevisaie* Cu-ATPase CCC2, a protein that is localized in the trans-Golgi region and transports Cu to Cu containing proteins, which represents an expansion of this subfamily compared with other fungi.
5. *RiCRD* putatively encodes a plasma membrane Cu-exporting ATPase that is expressed in the arbuscules and that is strongly up-regulated in the extraradical mycelia under Cu toxic conditions. RiCRD could have a dual role, one in Cu detoxification and the other in Cu transfer from the fungus to the periarbuscular space.
6. The decrease of arbuscular colonization under Cu-limiting conditions highlights the importance of Cu as an essential micronutrient for the development of the symbiosis.
7. The *Zea mays* genome harbours 12 genes putatively encoding heavy metal ATPases, being the expression of four of them (*ZmHMA3.3, ZmHMA5.1, ZmHMA5.2* y *ZmHMA5.3*) induced by Cu toxicity.
8. The higher Cu tolerance of mycorrhizal maize plants is partially due to the specific induction of two putative vacuolar ATPases, *ZmHMA3.3 y ZmHMA5.3*, both in roots and shoots of mycorrhizal plants. These transporters could play a dual role in shoot Cu protection, one by preventing root to shoot Cu translocation through its accumulation in root vacuoles and the other through vacuolar compartmentalization of the Cu that reaches the shoot.

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