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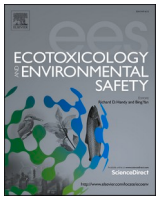
Ecotoxicology and Environmental Safety 234 (2022) 113390

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Impact of arbuscular mycorrhiza on maize P_{1B}-ATPases gene expression and ionome in copper-contaminated soils

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ARTICLE INFO

Edited by Dr Muhammad Zia-ur-Rehman

Keywords:
Arbuscular mycorrhiza
Copper toxicity
Maize
Heavy metal ATPases

ABSTRACT

Arbuscular mycorrhizal (AM) fungi, symbionts of most land plants, increase plant fitness in metal contaminated soils. To further understand the mechanisms of metal tolerance in the AM symbiosis, the expression patterns of the maize Heavy Metal ATPase (HMA) family members and the ionomes of non-mycorrhizal and mycorrhizal plants grown under different Cu supplies were examined. Expression of *ZmHMA5a* and *ZmHMA5b*, whose encoded proteins were predicted to be localized at the plasma membrane, was up-regulated by Cu in non-mycorrhizal roots and to a lower extent in mycorrhizal roots. Gene expression of the tonoplast ZmHMA3a and ZmHMA4 isoforms was up-regulated by Cu-toxicity in shoots and roots of mycorrhizal plants. AM mitigates the changes induced by Cu toxicity on the maize ionome, specially at the highest Cu soil concentration. Altogether these data suggest that in Cu-contaminated soils, AM increases expression of the HMA genes putatively encoding proteins involved in Cu detoxification and balances mineral nutrient uptake improving the nutritional status of the maize plants.

1. Introduction

Copper (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).

Plants need Cu at minimum concentrations of about 5 mg Kg^{-1} leaf dry biomass and encounter Cu toxicity when concentrations are higher than 20–30 mg Kg^{-1} (Marschner, 2012; Yruela, 2009). As a redox-active metal, excess Cu generates various reactive oxygen species via its participation in Haber-Weiss and Fenton reactions, causing damage to lipids, proteins and DNA (Linder, 1991). Toxicity can also result from displacement of other essential metals. A clear example is replacement of magnesium by Cu in the ribulose-1, 5-biphosphate-carboxylase/oxygenase resulting in loss of enzyme activity (van Assche and Clijsters, 1990). Usual symptoms of the oxidative stress and harmful interactions produced at the cellular level by Cu excess are an overall reduction of plant biomass, inhibition of root growth, chlorosis and necrosis.

Cu is normally present in soils as a trace element at concentrations ranging from 2.0–100 mg kg^{-1} (Adriano, 2005). However, over-use of Cu-containing pesticides and fungicides and release of industrial wastewater and residues 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 to people and animals since Cu can enter into the food-chain causing serious health problems (Cabral et al., 2015; Gohre and Paszkowski, 2006). In Europe, the maximum treshold level of Cu in soils for crop cultivation is 100 mg Kg^{-1} (MEF, 2007).

Plants have evolved certain strategies to avoid this toxicity. These strategies include reduction of Cu uptake and stimulation of Cu efflux through the plasma membrane, cytosolic chelation by metallothioneins or phytochelatins, sequestration in the vacuoles and activation of antioxidant systems (DalCorso et al., 2013; Pal and Rai, 2010; Viehweger, 2014). 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 (Chen et al., 2018; Smith and Read, 2008). AM fungi are obligate biotrophs that form mutualistic symbiosis with more than 70% of vascular plants, including several crops (Brundrett and Tedersoo, 2018). The fungus

Abbreviations: AM, arbuscular mycorrhiza; HMA, heavy metal ATPase; PCA, principal component analysis; Cu, copper.

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https://doi.org/10.1016/j.ecoenv.2022.113390
Received 2 September 2021; Received in revised form 12 November 2021; Accepted 3 March 2022
Available online 9 March 2022

provides the plant with low mobility mineral nutrients and water in exchange for plant-derived carbohydrates or lipids (Helber et al., 2011; Jiang et al., 2017; Lanfranco et al., 2018). This symbiosis also alleviates in the host plant the deleterious effects caused by biotic and abiotic stresses, such as salinity, drought and metal toxicity (Ferrol et al., 2016; Jung et al., 2012; Quiroga et al., 2019).

The mechanisms underlying the beneficial effect of AM fungi on plant fitness in metal contaminated soils are diverse. Metal toxicity alleviation by AM fungi could be due to 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 of the mycorrhizal plant or to the modulation of the plant metal response (Aloui et al., 2009; Ferrol et al., 2016; Pallara et al., 2013; Repetto et al., 2003). While several studies have 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; Merlos et al., 2016; Molina et al., 2020; Pallara et al., 2013), regulation of membrane transport proteins involved in Cu tolerance is still poorly understood.

Two major transporter families are involved in Cu transport in plants, the copper transporter (COPT-CTR) family and the P_{1B}-ATPases. Transporters of the COPT-CTR family govern plant Cu acquisition, while Cu efflux is mediated by P_{1B}-type ATPases (Migocka, 2015; Puig, 2014). 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 (Arguello et al., 2007). Plants have more HMAs than other organisms, with a wide variety of functions (Migocka, 2015; Williams and Mills, 2005). They have been studied at the genomic level *in Arabidopsis thaliana*, *Brassica napus*, *Sorghum bicolor, Glycine max*, *Oryza sativa* and *Zea mays* (Cao et al., 2019; Cobbett et al., 2003; Fang et al., 2016; Li et al., 2018; Williams and Mills, 2005; Zhiguo et al., 2018*)*. The well-characterised *A. thaliana* HMA family comprises eight members (Cobbett et al., 2003), rice has nine (Williams and Mills, 2005), and twelve HMA genes have been predicted in maize (Cao et al., 2019; Zhiguo et al., 2018). According to their substrate specificity, plant HMAs are phylogenetically classified into three major subgroups: the $P_{1B\text{-}1}$ subgroup clustering HMAs highly specific for Cu^{+}/Ag^{+} transport, the $P_{1B\text{-}2}$ subgroup of Zn/Cd/Pb-ATPases and the $P_{1B\text{-}4}$ subgroup including transporters with broad-specificity of divalent metal ions (Zn, Cd, Pb and Cu) (Li et al., 2018; Williams and Mills, 2005). Five HMAs have been related in *A. thaliana* with Cu transport: AtHMA5, AtHMA6 (also known as PAA1), AtHMA7 (also known as RAN1) and AtHMA8 (also known as PAA2) belonging to subgroup $P_{1B\text{-}1}$, and AtHMA1 of the subgroup $P_{1B\text{-}4}$ (Seigneurin-Berny et al., 2006; Williams and Mills, 2005). In rice, OsHMA4 has been associated with Cu accumulation in the vacuole (Huang et al., 2016) and the plasma membrane OsHMA5, OsHMA6 and OsHMA9 in Cu efflux from the cell (Deng et al., 2013; Lee et al., 2007; Wenli et al., 2020). Although the HMA gene family has been reported in maize, the role of the maize HMA family members in Cu homeostasis is not well understood yet.

With the aim of getting further insights into the role of the HMA transporters in Cu homeostasis and on their regulation by the AM symbiosis, we have analysed the expression patterns of the maize HMA family members in shoots and roots of non-mycorrhizal and mycorrhizal plants grown under different Cu supplies. Maize was chosen as host plant because it is one of the most important food grain crops. Therefore, a better understanding of the mechanisms of Cu accumulation in mycorrhizal maize plants is a first step for sustainable production of this staple cereal and for alleviation of health risks associated with the consumption of high-metal content agricultural products. Given that Cu stress could interfere with uptake of other mineral nutrients, we have also assessed the effects of Cu toxicity on the maize ionome and how these effects are modulated by the development of the symbiosis.

2. Materials and methods

2.1. Sequences analyses

Protein sequences of previously identified ZmHMAs were downloaded from Maize Genetics and Genomics database (https://www.maizegdb.org/) and used as a query for further analysis through Blastp searches in *Z. mays* datasets deposited on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Gramene (http://gramene.org/) and Aramemnon (http://aramemnon.uni-koeln.de/) websites. Full-length amino acid sequences were aligned with the orthologous sequences of *Arabidopsis thaliana*, *Oryza sativa, Brassica napus*, *Sorghum bicolor* and *Glycine max* 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 Servers (http://www.cbs.dtu.dk/services/TargetP/). Predictions of putative transmembrane helices were carried out with TOPCONS web server (http://topcons.cbr.su.se/).

2.2. Biological materials and growth conditions

The Cu-susceptible 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. Maize seeds provided by Semillas Fitó (Barcelona, Spain) were surface-sterilized with 70% ethanol for 10 min and with 10% sodium hypochlorite for 15 min. After 3 washes with distilled H_2O, seeds were transferred to a Petri dish containing a sterile wet filter paper and germinated for 3 days at 28 °C.

R. irregularis 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.

Soil was collected in the province of Granada 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. The plant growth substrate consisted of a mixture of steam-sterilized soil and peat (97:3, pH 6) supplemented with different Cu concentrations. Supplementation of the plant growth substrate with different Cu concentrations (0, 100, 250 mg Cu kg^{-1} soil) was performed by adding a $CuSO_4$ solution, mixing thoroughly and allowing to stabilize for 15 days.

2.3. Mycorrhizal inoculation

Mycorrhizal inoculation was performed by transferring the maize seedlings into 1 L pots containing the plant growth substrate supplemented with different Cu concentrations and 10% of the *R. irregularis* inoculum (mycorrhizal plants). Non-mycorrhizal plants were prepared by transferring the seedlings into pots containing the growth substrate, 10% of the substrate used to obtain the inoculum and a filtrate of the inoculum (< 20 μm) to provide the non-mycorrhizal soil microbiota.

2.4. Experimental design and growth conditions

A full factorial design with two factors: mycorrhization and copper availability 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} soil, referred as non-contaminated (NC), moderate (ModC) and high (HighC) level of soil Cu contamination, respectively. Seven replicates were considered, giving a total of 42 pots. 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. Three pools of two plants each were considered for nutrients content determination and gene expression analyses. Plants were grown during 8 weeks in a growth chamber at 25°C/18°C day/night, 16 h light photoperiod, relative humidity of 50–60%, photosynthetic photon flux density of 300 μmol$m^{-2}s^{-1}$ and watered three times per week with 25 ml H_2O.

2.5. Mycorrhizal colonization

Mycorrhizal colonization was assessed in root samples stained with trypan blue (0.05%) (Phillips and Hayman, 1970) using the magnified intersection method described by McGonigle et al. (1990). The abundance of the AM fungus in the roots was also estimated 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 analysed as an indicator of the functionality of the AM symbiosis (Glassop et al., 2005; Nagy et al., 2006).

2.6. Elemental concentration analyses

Shoots and roots of plants of all treatments were oven-dried at 70 °C for 48 h, and ground to a fine powder for nutrient (P, Ca, K, Mg, S, Na, B, Mn, Fe, Zn, Cu, Ni and Cr) content determinations. Tissue nutrient concentrations were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP 6500 Duo Thermo), using the Instrumentation Facility of the Estación Experimental del Zaidin, EEZ-CSIC, Granada, Spain.

2.7. Gene expression analyses

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 2.5 μM oligo $(dT)_{20}$ primer (Invitrogen), following the manufacturer's instructions. Gene transcript levels 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 0.2 μM of the corresponding specific primers listed in Table S1. All determinations were performed in at least three biological samples with the threshold cycle (Ct) determined in duplicate and in at least two independent PCR experiments. The amplification protocol used was 95 °C for 30 s, 38 cycles of [95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s] followed by a dissociation stage, except for *ZmHMA5.1* in which the annealing step was carried out at 63 °C 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).

2.8. Statistical analyses

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

3. Results

3.1. Maize P_{1B}-ATPases

As a first step to analyse the role of the HMAs on maize Cu tolerance and given that the 12 maize HMA genes previously described by Zhiguo et al. (2018) and Cao et al. (2019) were named differentially and that partial sequences were reported for some genes, we decided to revise the maize HMA gene family (Table S2). In our search we identified full-length sequences for ZmHMA8, according to the Cao et al. (2019) nomenclature, and for ZmHMA4, ZmHMA5b and ZmHMA9, according to the nomenclature of Zhiguo et al. (2018). Since the nomenclature of Zhiguo et al. (2018) fits better the nomenclature of the rice HMA orthologs, to provide a consensus nomenclature to the maize HMA genes we decided to name the maize HMAs according to Zhiguo et al. (2018), but including ZmHMA8, a maize HMA member not considered by these authors. As Zhiguo et al. (2018), the *ZmHMA12* gene reported by Cao et al. (2019) has neither been considered in this study because the encoded protein is too small, 400 amino acids, and identical to the C terminal part of ZmHMA9 (Fig. S1). The revised nomenclature and structural features of the ZmHMAs is summarised in Table S2.

To explore the phylogenetic relationships of the plant HMA proteins, an unrooted phylogenetic tree was constructed with the deduced amino acid sequences of the previously identified HMA genes of *A. thaliana*, *O. sativa*, *B. napus*, *S. bicolor, G. max* and *Z. mays.* Based on their relative positions in the tree, they were classified within the three groups of the HMA families: $P_{1B\text{-}1}$ subgroup of Cu^+/Ag^+-ATPases (clusters III, IV, V and VI), $P_{1B\text{-}2}$ subgroup of Zn/Cd/Pb-ATPases (cluster II) and $P_{1B\text{-}4}$ subgroup including HMA1-like proteins with broad-specificity of metals (cluster I) (Fig. 1).

3.2. ZmHMA genes are differentially regulated in roots and shoots

To characterize the maize HMA gene family under our experimental conditions, expression of the 12 *ZmHMA* genes was assessed in roots and shoots of control plants by RT-qPCR. Transcript levels of *ZmHMA1, ZmHMA3b, ZmHMA5a, ZmHMA7* and *ZmHMA8* were significantly higher in shoots than in roots, whereas transcript levels of *ZmHMA5b* and *ZmHMA9* were higher in roots. No significant differences in transcript levels of *ZmHMA2*, *ZmHMA3a, ZmHMA4* and *ZmHMA6* were found between roots and shoots. *ZmHMA10* was not expressed in our experimental conditions or its expression level was below the detection limit (Fig. 2).

3.3. Effect of Cu availability and mycorrhiza on ZmHMAs gene expression

To test the effect of Cu toxicity and mycorrhiza on HMAs gene expression, their expression was analysed in shoots and roots of non-inoculated (NM) and *R. irregularis*-inoculated (MYC) maize plants grown in soils non-supplemented or supplemented with 100 or 250 mg Kg^{-1} soil. As expected, Cu stress had a negative impact on plant biomass and this growth inhibition was significantly reduced in plants colonised by *R. irregularis* (Table S3 and Fig. S2). In non-mycorrhizal plants, shoot biomass was reduced by 26% at 100 mg Kg^{-1} soil and by 53% at 250 mg Kg^{-1} soil, whereas in mycorrhizal plants it was reduced by 13% and 35%, respectively. Root biomass was only inhibited at the highest Cu concentration, being this inhibition 1.7-fold lower in mycorrhizal plants.

Expression of the *ZmHMA* genes was differentially regulated by mycorrhiza and Cu factors (Figs. 3 and 4 and Table S4). Shoot *ZmHMA*s expression was not affected by Cu toxicity in non-mycorrhizal plants

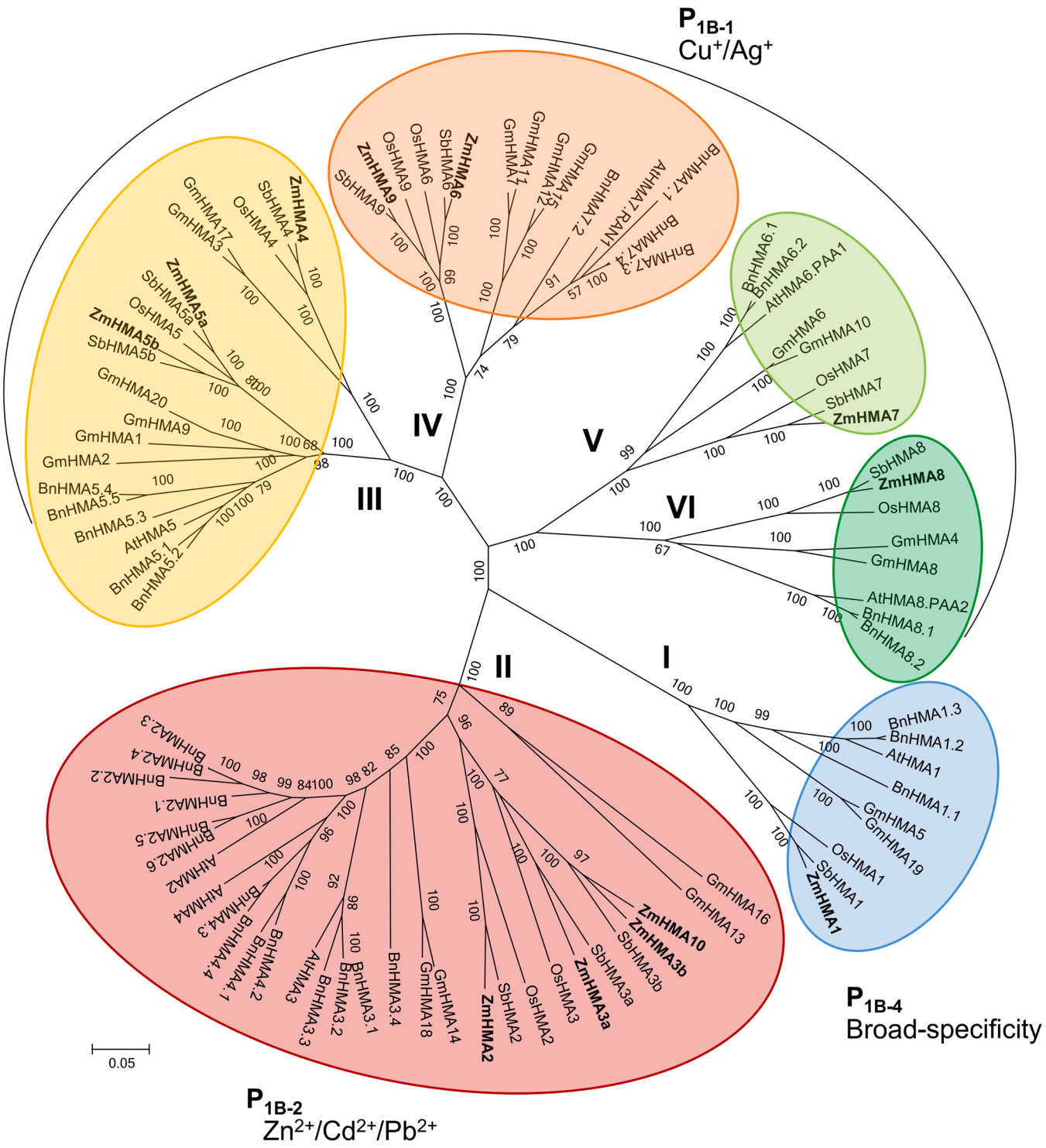

Fig. 1. Phylogenetic relationships of plant HMA proteins. The unrooted Neighbor-joining tree was generated using MEGA v.6 with 1000 bootstrap replicates. *Zea mays* HMA proteins are in bold. Organisms: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Gm, *Glycine max*; Os, *Oryza sativa*; Sb, *Sorghum bicolor*; Zm, *Z. mays*. Bootstrap values above 70 and supporting a node used to define a cluster are indicated.

(Fig. 3). However, shoot *ZmHMA4* and *ZmHMA9* expression were up-regulated in mycorrhizal plants grown in the soil supplemented with 100 mg Cu Kg^{-1} soil and an increase in shoot *ZmHMA3a* expression was observed at the highest Cu soil concentration. The interaction between mycorrhiza and Cu was only significant for shoot *ZmHMA3a* and *ZmHMA4* (Table S4).

In non-mycorrhizal roots, expression of *ZmHMA5a* and *ZmHMA5b*, whose gene products were predicted to localize to the plasma membrane (Table S2), was up-regulated by Cu toxicity (Fig. 4). While *ZmHMA5a* expression increased in roots of plants grown in soils contaminated with 100 and 250 mg Cu Kg^{-1} soil, *ZmHMA5b* expression only increased at the highest Cu concentration. In contrast, Cu toxicity decreased *ZmHMA6*, *ZmHMA8* and *ZmHMA9* expression in non-mycorrhizal roots, being *ZmHMA6* down-regulated only in plants grown in soils supplemented with 100 mg Cu Kg^{-1} soil, *ZmHMA8* at the highest Cu concentration and *ZmHMA9* at both Cu concentrations. Development of the

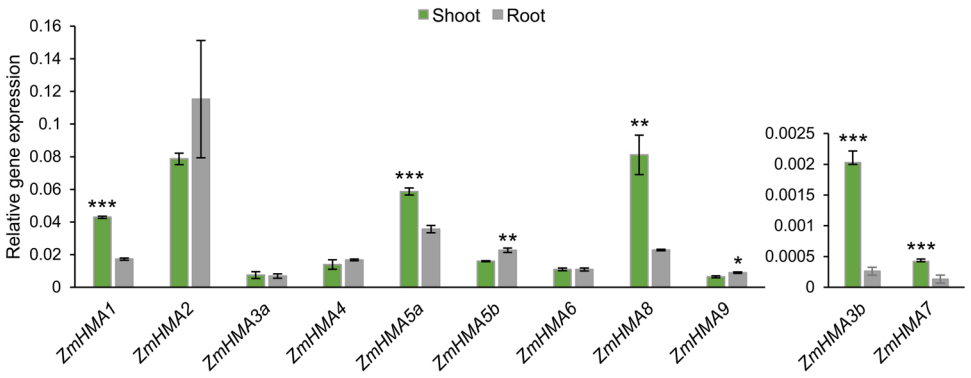

Fig. 2. *ZmHMA*s expression levels in different plant tissues. Gene expression patterns of *ZmHMA* genes in shoots and roots of non-mycorrhizal plants grown in the non-contaminated soil (control plants). Relative gene expression levels were calculated by the $2^{-\Delta CT}$ method using the elongation factor 1α of *Z. mays* (*ZmEF1α*) as a normalizer. Bars represent standard error. Asterisks show statistically significant differences (* $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$) between gene expression in shoots and roots according to the Student's *t*-test.

symbiosis in the non-contaminated soil increased root *ZmHMA3b* transcript levels and down-regulated *ZmHMA8* and *ZmHMA9* gene expression. In mycorrhizal roots, Cu toxicity increased the expression of *ZmHMA3a, ZmHMA4, ZmHMA5a* and *ZmHMA5b* (Fig. 4). Interestingly, in the *R. irregularis*-colonised roots *ZmHMA5a* expression was only increased at the highest Cu concentration and *ZmHMA5b* expression was induced to a lower extent than in the non-inoculated roots (Fig. 4). Altogether these data indicate that the P_{1B-1} subgroup of HMAs has the highest impact in Cu detoxification in maize plants.

3.4. Effect of Cu and mycorrhiza on the maize ionome

Taking into account the role of the AM symbiosis on plant mineral nutrition and that Cu stress could interfere with the uptake of other mineral nutrients, the ionome of plants of all treatments was analysed by ICP-MS. Despite most studies report the mineral nutrient concentrations, here we decided to examine nutrient contents to get information on differences in uptake and distribution. Most of the nutrient contents responded significantly to mycorrhiza and/or Cu factors, and the interaction between them was significant for shoot phosphorus, potassium, and magnesium contents, and for root Cu content (Figs. S3 and S4 and Table S5). PCA analyses of the data revealed a strong impact of the Cu treatment on shoot and root nutrient accumulation in both mycorrhizal and non-mycorrhizal plants (Figs. 5 and 6). The major changes induced by Cu toxicity were an increase in shoot and root Cu and sulfur contents, a decrease in shoot and root potassium, phosphorus and calcium contents, a decrease of shoot boron, magnesium, sodium and zinc contents and an increase of root manganese content (Fig. 6, S3 and S4). In the non-contaminated soil, shoot and root nutrient content profiles of non-mycorrhizal and mycorrhizal plants were quite similar. Under these conditions, an increase in potassium content and a decrease in manganese content were detected in shoots of mycorrhizal plants compared to non-mycorrhizal plants, while no significant differences were detected at the root level. Although no significant differences were observed in P content of mycorrhizal and non-mycorrhizal plants, symbiotic Pi transport was active given that the mycorrhiza-specific phosphate transporter gene *ZmPht1;6* was expressed in the mycorrhizal roots (Fig. 7).

The ionomes of the mycorrhizal and non-mycorrhizal plants grown in the contaminated soils were different, especially at the highest Cu concentration. In the contaminated soils, the symbiosis decreased shoot and root Cu concentrations, increasing, therefore, maize Cu tolerance (Table S3). However, shoot and root Cu accumulation increased in mycorrhizal plants grown at the highest Cu concentration because of their higher biomass (Figs. S3 and S4). The nutrient profile of mycorrhizal plants grown at 250 mg Cu Kg^{-1} soil was similar to the nutrient profiles of non-mycorrhizal and mycorrhizal plants grown in soils supplemented with 100 mg Cu Kg^{-1} soil (Figs. 5 and 6). In general, mycorrhizal plants grown at the highest Cu concentration displayed an improved nutritional status compared to their non-mycorrhizal counterparts, specially shoot and root nutrients within Clusters II and III, respectively (Figs. 5, 6, S3 and S4). In contrast with what was observed in the non-contaminated soil, mycorrhizal plants grown in the Cu contaminated soils had higher phosphorus content (Fig. 6, S3 and S4). Expression of the marker gene of symbiotic Pi transport *ZmPht1;6* was slightly but significantly higher in roots grown at 100 mg Cu Kg^{-1} soil than in control roots and decreased at the highest Cu concentration. However, mycorrhizal colonization, as estimated by the arbuscular colonization percentage and the expression level of the *R. irregularis* elongation factor 1α (*RiEF1α*), was only affected when plants were grown in the soil supplemented with 250 mg Cu Kg^{-1} soil, being highly inhibited at this Cu concentration (Fig. 7).

4. Discussion

Different mechanisms have been proposed to explain the protective effect of AM fungi to Cu toxicity. In this manuscript we show for the first time that in Cu contaminated soils development of the symbiosis regulates the expression of some members of the maize HMA family, proteins playing a key role in root to shoot metal translocation and in vacuolar metal sequestration. The finding that AM mitigate the negative impact of Cu stress on plant biomass agrees with previous observations in maize and other plant species (Cicatelli et al., 2010; Pallara et al., 2013; Merlos et al., 2016).

Our gene expression data revealed that in plants grown in the non-contaminated soil *ZmHMA2*, *ZmHMA4* and *ZmHMA6* were constitutively expressed, *ZmHMA8* was more highly expressed in shoots than in roots and that *ZmHMA5b* and *ZmHMA9* were more highly expressed in roots. This expression pattern agrees with what has been previously found in the maize gene expression atlas (Cao et al., 2019; Hoopes et al., 2019; Stelpflug et al., 2016). These data confirm previous observations in various plant species showing that the HMA genes are differentially expressed in different tissues and developmental stages (Fang et al., 2016; Li et al., 2018; Migeon et al., 2010).

ZmHMA1, ZmHMA7 and *ZmHMA8,* whose encoded proteins were predicted to have a 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) were proposed to function sequentially in Cu transport over the plastid envelope and the thylakoid membrane, respectively. Both isoforms are crucial for Cu delivery to Cu containing proteins in the chloroplast, such as the stromal Cu/Zn superoxide dismutase 2 (CSD2) and plastocyanin (Abdel-Ghany et al., 2005; Catty et al., 2011; Cohu and Pilon, 2007; Shikanai et al., 2003). AtHMA1 is a Ca^{2+}/ heavy metal ATPase involved in the transport of multiple cations between the chloroplast and the cytosol (Boutigny et al., 2014; Kim et al., 2009; Moreno et al., 2008). Shoot *ZmHMA1* expression was neither regulated by AM nor by Cu toxicity. However, the HMA1-like P_{1B-4} ATPase *GmHMA19* of soybean was found to be up-regulated by AM under cadmium toxicity

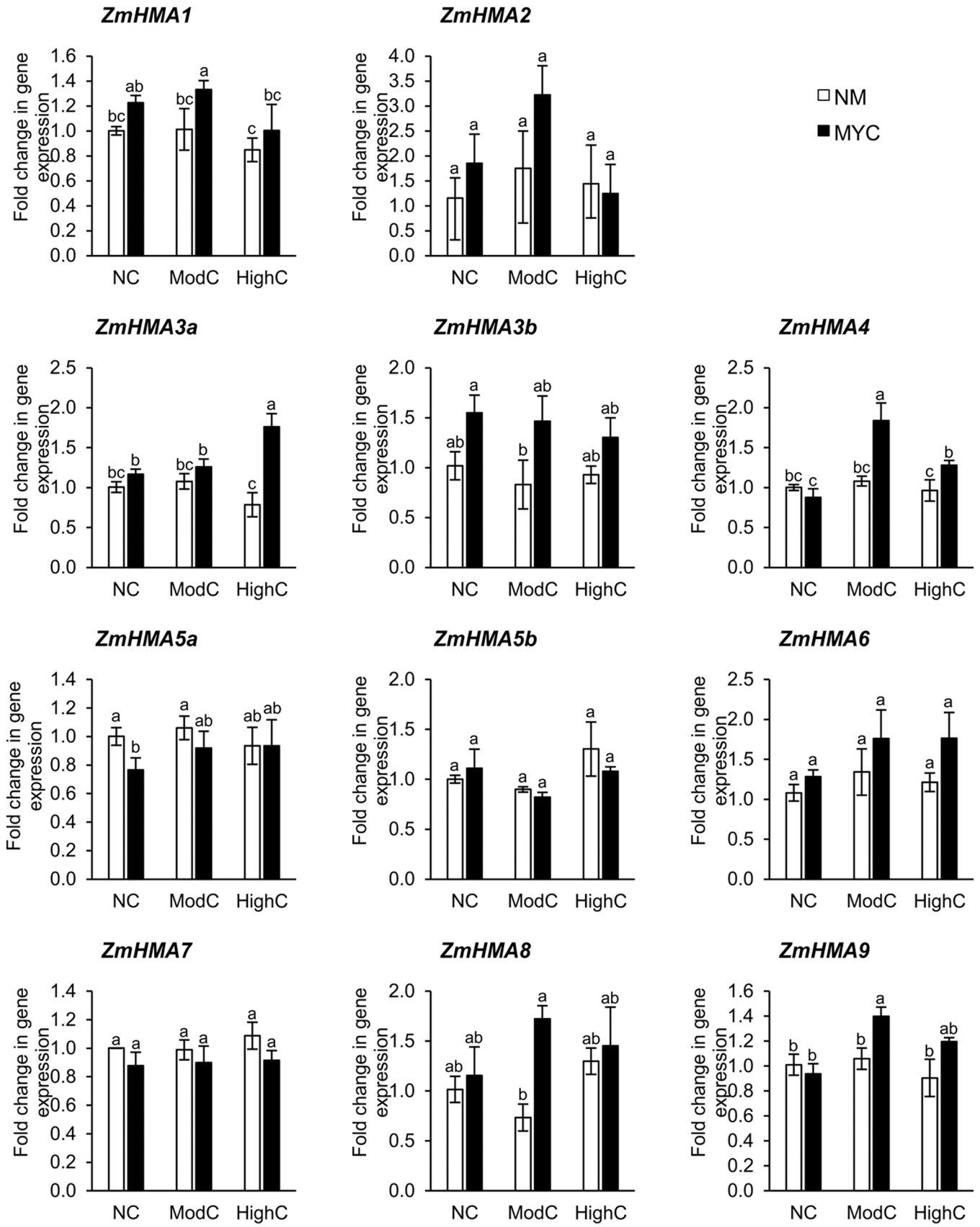

Fig. 3. Effect of Cu toxicity and mycorrhizal inoculation on shoot *ZmHMA* gene expression. Non-mycorrhizal (NM) and mycorrhizal (MYC) plants were grown in soils supplemented with 0 (non-contaminated, NC), 100 (moderate, ModC) or 250 mg Cu (high, HighC) Kg^{-1} soil. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method using the elongation factor 1α of *Z. mays* (*ZmEF1α*) as a normalizer. Bars represent standard error; different letters indicate significant differences ($P < 0.05$) between treatments according to the Duncan's test.

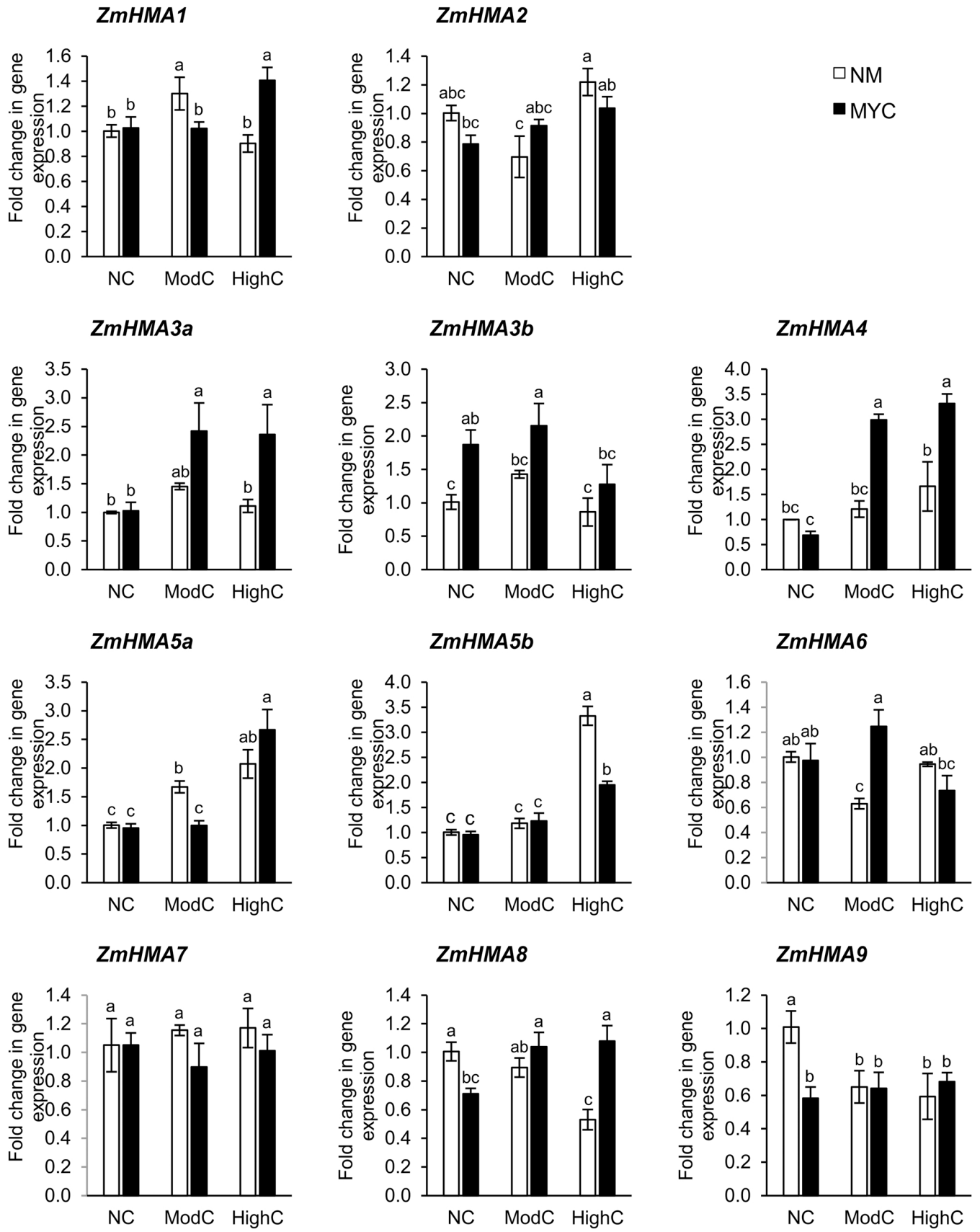

Fig. 4. Effect of Cu toxicity and mycorrhizal inoculation on root *ZmHMA* gene expression. Non-mycorrhizal (NM) and mycorrhizal (MYC) plants were grown in soils supplemented with 0 (non-contaminated, NC), 100 (moderate, ModC) or 250 mg Cu (high, HighC) Kg^{-1} soil. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method using the elongation factor 1α of *Z. mays* (*ZmEF1α*) as a normalizer. Bars represent standard error; different letters indicate significant differences ($P < 0.05$) among treatments according to the Duncan's test.

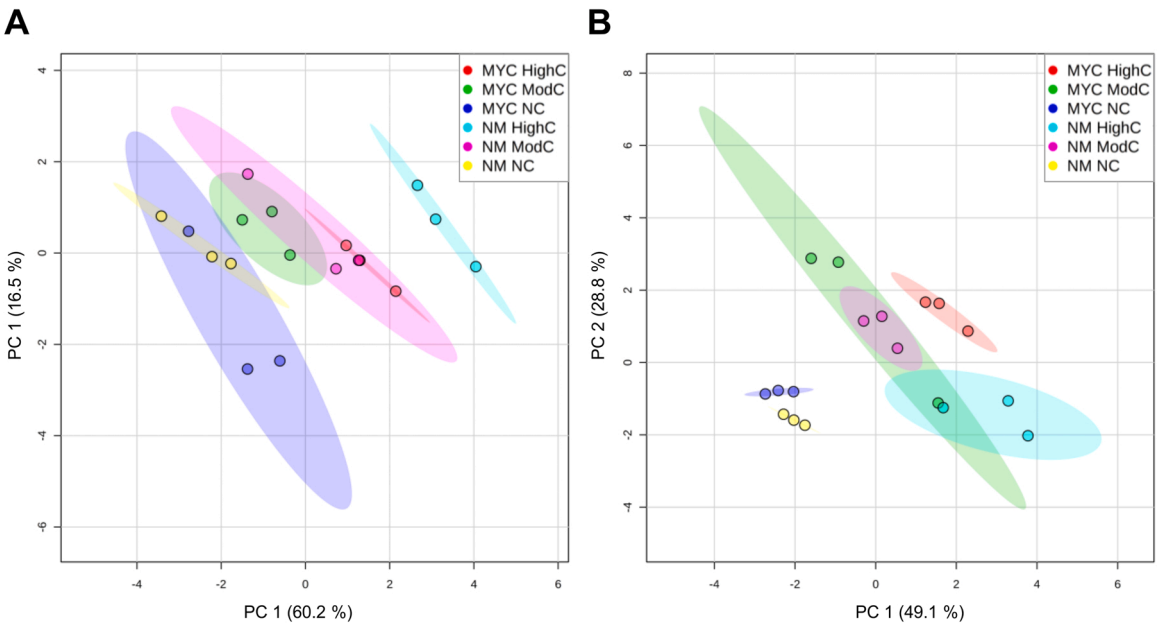

Fig. 5. Nutrient content pattern reorganization of maize plants subjected to the different treatments. Principal Component Analysis (PCA) of nutrient contents in shoots (A) and roots (B) of non-mycorrhizal (NM) and mycorrhizal (MYC) plants grown in soils supplemented with 0 (non-contaminated, NC), 100 (moderate, ModC) or 250 mg Cu (high, HighC) Kg^{-1} soil. Each color represents one treatment: in dark blue: MYC NC, in yellow: NM NC, in green: MYC ModC, in pink: NM ModC, in red: MYC HighC and in light blue: NM High C. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

(Cui et al., 2019). Interestingly, Cu toxicity did not affect shoot expression levels of any of the chloroplast tagged HMAs. However, *ZmHMA8* expression was down-regulated by Cu toxicity in roots of non-mycorrhizal plants, probably to preserve root plastid function under these conditions.

On the other hand, ZmHMA6 and ZmHMA9 are phylogenetically close to OsHMA6 and OsHMA9, respectively, and cluster in the same subgroup than AtHMA7/RAN1. *ZmHMA9* was highly expressed in roots while no significant differences in *ZmHMA6* expression between shoots and roots were detected. This expression pattern was also observed for *OsHMA6* and *OsMHA9* (Wenli et al., 2020). These rice isoforms were shown to be plasma membrane Cu efflux proteins related with Cu tolerance (Lee et al., 2007). However, AtHMA7/RAN1 was proposed to be localized in the post-Golgi compartment and was shown to participate in Cu delivery to ethylene receptors, proteins that need Cu as a cofactor (Hirayama et al., 1999; Keunen et al., 2016; Rodriguez et al., 1999; Woeste and Kieber, 2000). Down-regulation by Cu toxicity of root *ZmHMA6* and *ZmHMA9* expression indicates that their encoded proteins are not involved in Cu detoxification. Further studies are required to determine ZmHMA6 and ZmHMA9 cellular and subcellular localization and to elucidate if they could be involved in ethylene signalling, as it has been described for AtHMA7.

Up-regulation by Cu toxicity of *ZmHMA5a* and *ZmHMA5b* in non-mycorrhizal and mycorrhizal roots suggests a role for these ATPases in Cu detoxification. Since the proteins encoded by *ZmHMA5a* and *ZmHMA5b* 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 distribution 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 *ZmHMA5a* and *ZmHMA5b* transcripts accumulate in a Cu-dependent manner, the finding that in mycorrhizal roots *ZmHMA5a* was only up-regulated at the highest Cu concentration and that *ZmHMA5b* 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 and extraradical mycelium than in root cells of a mycorrhizal root (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.

ZmHMA4 expression is up-regulated by Cu toxicity in mycorrhizal tissues and is closely related to the *O. sativa* tonoplast Cu transporter OsHMA4 that transports Cu ions from the cytosol to the vacuoles preventing its toxicity (Huang et al., 2016). *OsHMA4* and the two vacuolar HMAs of *Cucumber sativus CsHMA5.1* and *CsHMA5.2* are mainly expressed in roots and contribute to metal detoxification in the root vacuoles reducing, therefore, root to shoot Cu translocation (Huang et al., 2016; Migocka et al., 2015). However, *ZmHMA4* 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). As proposed by these authors, our data suggest that ZmHMA4 could play a dual role in shoot Cu protection: (i) an indirect role by preventing root to shoot Cu translocation through its accumulation in the root vacuole and (ii) a direct role through vacuolar compartmentalization of the Cu that reaches the shoot. It is noteworthy that Cu toxicity up-regulates *ZmHMA4* expression only in roots and shoots of mycorrhizal plants. These data suggest that under Cu toxic conditions AM 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 *ZmHMA4* expression in shoots of mycorrhizal plants indicates that development of the symbiosis induces a systemic effect on the plant detoxification mechanisms.

In addition to these well-characterized Cu-ATPases, ZmHMA3a, that is included in the P_{1B-2} subgroup, might be also involved in Cu homeostasis. *ZmHMA3a* was highly up-regulated by Cu toxicity in roots and shoots of mycorrhizal plants. As shown for its closest ortholog in rice

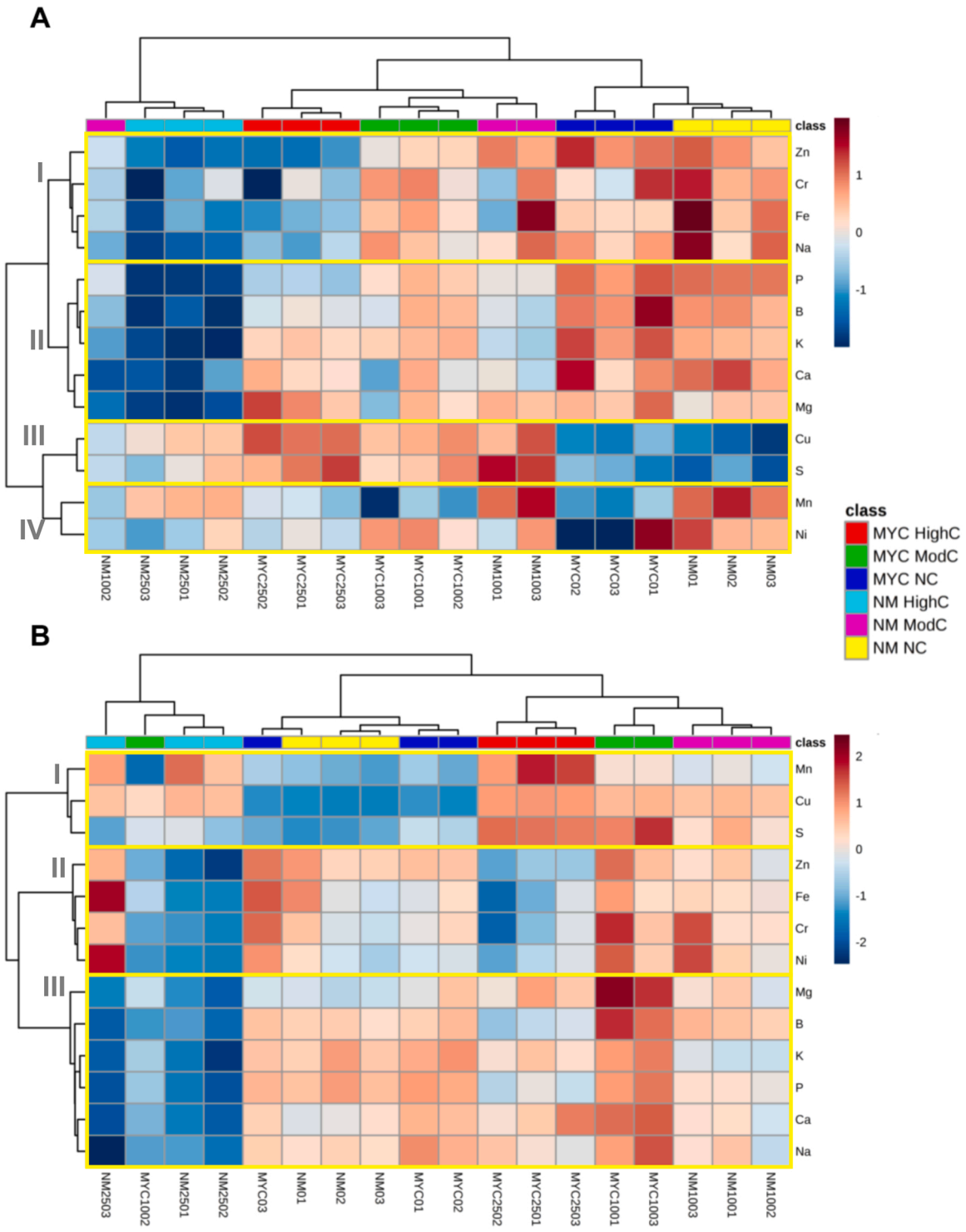

Fig. 6. Heat maps and clustering of the nutrient content of maize plants subjected to the different treatments. Nutrient contents in shoots (A) and roots (B) of non-mycorrhizal (NM) and mycorrhizal (MYC) plants grown in soils supplemented with 0 (non-contaminated, NC), 100 (moderate, ModC) or 250 mg Cu (high, HighC) Kg^{-1} soil. Treatments are represented in different colors: in dark blue: MYC NC, in yellow: NM NC, in green: MYC ModC, in pink: NM ModC, in red: MYC HighC and in light blue: NM High C. Based on nutrient accumulation patterns observed, four or three groups were identified in shoots an in roots, respectively. Cluster II in shoots and Cluster III in roots include nutrients whose content (mg $plant^{-1}$) in plant tissues were higher in mycorrhizal plants grown in Cu contaminated soils compared to non-mycorrhizal, especially at the highest Cu concentration. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

OsHMA3, ZmHMA3a is probably tagged to the tonoplast and might prevent Cu toxicity by sequestering Cu in root and shoot vacuoles. Although HMA3-like proteins have been mostly related with Zn^{2+} and/or Cd^{2+} transport, including rice OsHMA3 (Liu et al., 2017; Miyadate et al., 2011; Morel et al., 2009), the capability of ZmHMA3a to transport other metals cannot be discarded.

Regarding the ionomic data, the finding that the nutrient profile of mycorrhizal plants grown at 250 mg Cu Kg^{-1} soil was similar to the

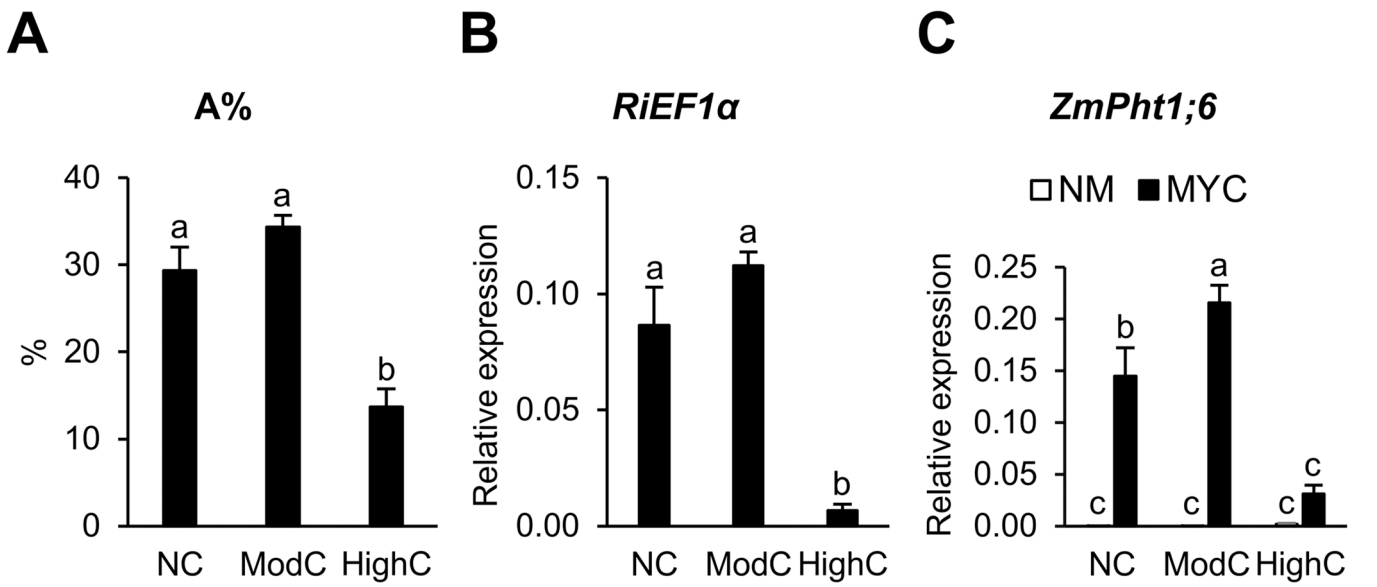

Fig. 7. Effect of Cu toxicity on mycorrhizal colonization. (A) Percentage of arbuscules (A%). Gene expression patterns of the *R. irregularis* elongation factor 1α *RiEF1α* (B) and the *Z. mays* mycorrhiza-induced phosphate transporter *ZmPht1;6* (C) in mycorrhizal (MYC) roots grown in soils supplemented with 0 (non-contaminated, NC), 100 (moderate, ModC) or 250 mg Cu (high, HighC) Kg^{-1} soil. Relative gene expression was calculated using the $2^{-\Delta CT}$ method with *EF1α* of maize as internal control. Bars represent standard error. Different letters indicate significant differences ($P < 0.05$) among treatments according to the Duncan's test.

nutrient profiles of plants grown in soils supplemented with 100 mg Cu Kg^{-1} soil indicates that the AM symbiosis mitigates the changes induced by the highest Cu soil concentration on the maize ionome. Therefore, the increased Cu tolerance of the mycorrhizal plants is partially due to the role of the AM fungus on plant host nutrition, preventing the alterations induced by Cu toxicity on nutrient homeostasis. These data also indicate that the beneficial effect of the symbiosis on plant performance is more notorious at the highest Cu soil concentration and that under these conditions the plant shows a higher mycorrhizal dependence. Comparisons between Cu concentrations and uptake in mycorrhizal and non-mycorrhizal plants grown in the contaminated soils revealed that the decrease induced by mycorrhizal colonization in tissue Cu concentrations is partially due to a dilution effect. Interestingly, while no significant differences were observed between Cu uptake of non-mycorrhizal and mycorrhizal plants grown at 100 mg Cu Kg^{-1} soil, Cu uptake was higher in mycorrhizal plants grown in soils with the highest levels of contamination. These data support the view that the use of mycorrhizal plants could increase the efficiency of phytoremediation strategies of metal contaminated soils.

Among the changes induced by Cu toxicity in mineral nutrient contents, and besides the expected increase in Cu content, a decrease in K and Ca contents has been reported in different plant species (Andrade et al., 2010; Maksymiec and Baszyński, 1998; Murphy et al., 1999). However, variations in other nutrients such as, Mg, S, Fe and Zn, depends on the plant species (Ke et al., 2007; Lequeux et al., 2010). The decrease in nutrient uptake under Cu toxic conditions has been attributed to the inhibition of root growth induced by Cu toxicity, which results in a diminished exploration of the soil by roots (Marschner, 2012). In our experimental system, a decrease in shoot and root K and Ca contents was also observed in the Cu-contaminated soil, effects that were reverted in the tissues of mycorrhizal plants. A similar trend was observed for P accumulation in the Cu contaminated soils. Although the major effect of AM on plant mineral nutrition is an increase in P acquisition, a clear contribution of the AM symbiosis on K and Ca uptake has been reported (Ferrol et al., 2019; Garcia and Zimmermann, 2014; Liu et al., 2002; Wipf et al., 2019). The higher K, Ca and P contents of the mycorrhizal plants grown under Cu toxicity is likely due to the capability of the AM fungal extraradical myelium to explore a volume of soil not accesible to the root and to transfer nutrients present in these soil zones to the plant.

Interestingly, no significant differences were found between the total P contents of non-mycorrhizal plants grown in the non-contaminated soil. However, the high expression levels of the mycorrhiza-specific P transporter *ZmPht1;6* detected in the mycorrhizal roots indicates that the symbiosis was functional and that the plant is receiving phosphorus through the mycorrhizal pathway. Our data are in agreement with previous observations in the tomato-*R. irregularis* association (formerly, *Glomus intraradices*) (Smith et al., 2003). Physiological and molecular studies have demonstrated that during AM symbiosis the plant changes its acquisition strategy and favours Pi acquisition from the AM fungus over acquisition through its epidermal cells (Sawers et al., 2017; Smith et al., 2003; Yang et al., 2012). Therefore, our data indicate that in the non-contaminated soil the mycorrhizal pathway is "hidden" and that activation of the mycorrhizal phosphorus uptake pathway inhibits the direct uptake pathway through its epidermal cells. This might be also the case for the acquisition of the other nutrients, which would explain the similar nutrient profiles of non-mycorrhizal and mycorrhizal plants grown in the non-contaminated soil. Our data showing a decreased Mn uptake in mycorrhizal roots agrees with previous observation in other plant species (Liu et al., 2000; Correa et al., 2014). Lower Mn uptake in mycorrhizal plants has been attributed to a decreased presence of Mn-reducers as a consequence of the changes induced by mycorrhizal colonization in the activity of the rhizosphere microorganisms (Kothari et al., 1991) or to fungal Mn retention (Correa et al., 2014).

5. Conclusion

In conclusion, our results strongly suggest a role in Cu detoxification for ZmHMA5a and ZmHMA5b, probably acting as Cu efflux plasma membrane pumps, and for ZmHMA3a and ZmHMA4, probably sequestering Cu ions in shoot and root vacuoles reducing Cu translocation to aerial tissues (Fig. 8). Enhanced expression of the two putative tonoplast transporters in mycorrhizal plants under Cu toxicity indicates that AM fungi up-regulate the intrinsic mechanisms of metal detoxification. Our data also show that AM prevents the alterations induced by Cu toxicity on nutrient homeostasis. Further functional characterization of the Cu-induced *ZmHMA* genes is required to better understand their role in Cu accumulation and in alleviation of metal toxicity by AM. These data

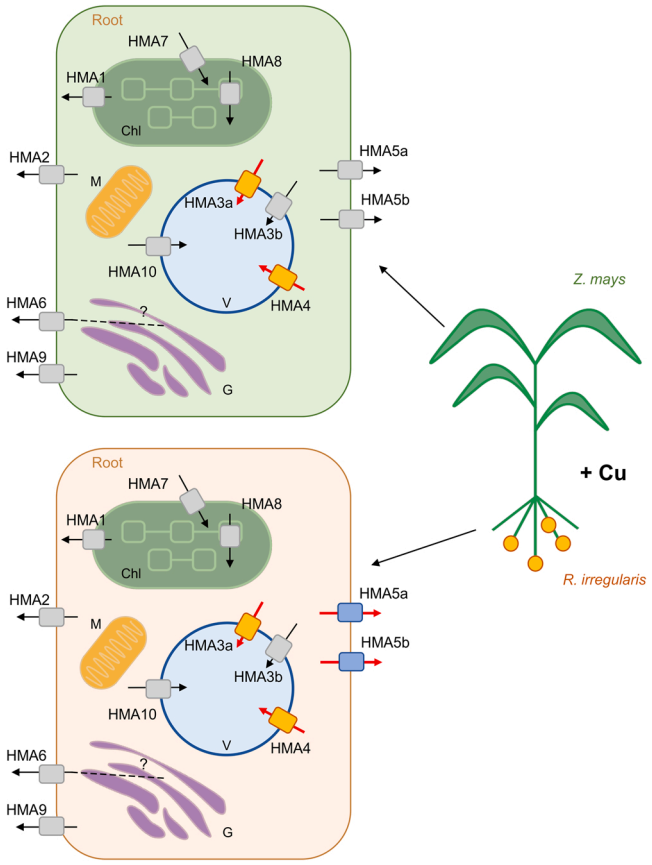

Fig. 8. Schematic representation of the major changes induced by Cu in *ZmHMAs* expression in maize mycorrhizal and non-mycorrhizal plants. Red arrows represent up-regulated *ZmHMAs*. Orange transporters: genes up-regulated by Cu in mycorrhizal plants; blue transporters: *ZmHMAs* induced by Cu in both non-mycorrhizal and mycorrhizal plants; grey transporters: *ZmHMAs* not induced by Cu. Chl: chloroplast; V: vacuole; G: golgi. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

could be used for the development of molecular markers for the selection of low Cu accumulating maize cultivars and to minimize the health risks associated with the growth of this staple cereal in agricultural soils previously treated with Cu-based pesticides.

CRediT authorship contribution statement

Tamara Gómez-Gallego: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft & editing. **Ascensión Valderas:** Investigation, Resources. **Diederik van Tuinen:** Methodology, Supervision, Writing – review & editing. **Nuria Ferrol:** Conceptualization, Methodology, Supervision, Funding acquisition, Project administration, Writing – original draft, editing.

Declaration of Competing Interest

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

Acknowledgements

Projects AGL2015-67098-R and RTI2018-098756-B-I00 funded by MCIN/AEI /10.13039/501100011033/ and FEDER Una manera de hacer Europa. Tamara Gómez-Gallego was supported by a PhD contract (FPI) from MINECO, Spain.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113390.

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