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The function of the *Medicago truncatula* ZIP transporter MtZIP14 is linked to arbuscular mycorrhizal fungal colonization

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Abstract

Soil micronutrient availability, including zinc (Zn), is a limiting factor for crop yield. Arbuscular mycorrhizal (AM) fungi can improve host plant growth and nutrition through the mycorrhizal pathway of nutrient uptake. Although the physiology of Zn uptake through the mycorrhizal pathway is well established, the identity of the related molecular components are unknown. Here, RNA-seq analysis was used to identify genes differentially-regulated by AM colonization and soil Zn concentration in roots of *Medicago truncatula*. The putative Zn transporter gene *MtZIP14* was markedly up-regulated in *M. truncatula* roots when colonized by *Rhizophagus irregularis*. MtZIP14 restored yeast growth under low Zn availability. Loss-of-function mutant plants (*mtzip14*) had reduced shoot biomass compared to the wild-type when colonized by AM fungi and grown under low and sufficient soil Zn concentration; at high soil Zn concentration, there were no genotypic differences in shoot biomass. The vesicular and arbuscular colonization of roots was lower in the *mtzip14* plants regardless of soil Zn concentration. We propose that MtZIP14 is linked to AM colonization in *M. truncatula* plants, with the possibility that *MtZIP14* function with AM colonization is linked to plant Zn nutrition.

KEYWORDS
arbuscular mycorrhizal fungi, nutrient uptake, zinc

1 | INTRODUCTION

Zinc (Zn) is an essential cofactor for >300 enzymes in plants, making it critical for processes such as carbon fixation, transcription and production of ATP (Broadley et al., 2007; Marschner, 2012). It is also an essential micronutrient for humans and Zn deficiency is the fifth leading risk factor for disease in developing countries with high mortality (Brown & Wuehler, 2000). Zn is taken up at the plant–soil interface in its divalent form Zn^{2+} by the Zn-regulated iron-regulated transporter-like protein (ZIP) family, which also have a role in the transport of other transition metals (Guerinot, 2000), and also by yellow stripe-like proteins when Zn is complexed with phytosiderophores in monocots (Borrill et al., 2014). ZIP transporters are involved in cellular Zn homeostasis (Grotz & Guerinot, 2006; Milner et al., 2013) and the plant response to Zn deficiency (Grotz et al., 1998; Pedas et al., 2009), while overexpression of ZIPs can lead to increased tissue Zn concentrations (Ramegowda et al., 2013; Tiong et al., 2014). Characteristics of most ZIP transporters include eight predicted transmembrane-spanning α-helices, and a hydrophilic variable region between helix III and IV that contains a potential metal-binding domain (Guerinot, 2000). In the model legume *Medicago truncatula*, 16 predicted ZIP transporters have been identified through phylogenetic analysis (Abreu et al., 2017) and four of those have been characterized for Zn transport function by expression in the yeast mutant ZHY3 that lacks Zn transporters (López-Millán et al., 2004); however, besides MtZIP6 which is involved in Zn uptake in the rhizobial symbiosis (Abreu et al., 2017), the specific roles of all of the remaining ZIPs *in planta*, are unknown.

The majority (>80%) of terrestrial plant species, including most important crops, form associations with arbuscular mycorrhizal (AM) fungi. Resource exchange is critical to the symbiotic association and typically involves trade of inorganic nutrients from the fungus with carbon resources from the plant (Garcia et al., 2016). The primary benefits of colonization by AM fungi is improvement in plant growth and nutrition, particularly of nitrogen (N), phosphorus (P) and Zn (Cavagnaro, 2008; Smith & Read, 2008). Managed effectively, AM fungi provide a tool for improved crop Zn nutrition in the field, particularly on Zn-deficient soils (Ercoli et al., 2017). Radioisotope tracing studies have demonstrated that the AM fungus *Rhizophagus irregularis* can contribute as much as 25% of shoot Zn uptake in tomato plants, 24% of grain Zn in wheat and 12% in barley (Coccina et al., 2019; S. Watts-Williams, Smith et al., 2015).

Considerable progress has been made toward identifying the components involved in plant P and N nutrition facilitated by AM associations (Ferrol et al., 2019; Javot et al., 2007; Wang et al., 2020), and an AM-specific plant Cu transporter has been identified (Senovilla et al., 2020). To fully exploit the AM symbiosis for improved agricultural outcomes (i.e., crop quantity and quality, biofortification), it is essential that these molecular components are identified (Ruytinx et al., 2019; Schroeder et al., 2013).

While an AM fungal transporter that facilitates Zn regulation in extraradical hyphae has been identified (González-Guerrero et al., 2005), no plant Zn transporter has been identified that is involved in the AM association (Ferrol et al., 2016; Ruytinx et al., 2019). It has been postulated that an, as yet, unidentified Zn transporter is exclusively located on the plant-derived periarbuscular membrane (PAM) present in AM-colonized root cortical cells, responsible for the import of Zn^{2+} supplied by the fungus (Ferrol et al., 2019). To begin to uncover the identity of such a Zn transporter, there were several research aims for the present work:

1. Conduct a transcriptomic screen of *R. irregularis* inoculated versus non-inoculated roots at different soil Zn concentrations to identify highly up-regulated Zn-related genes.
2. Characterize the primary candidate gene through subcellular localization and expression in a heterologous yeast system.
3. Determine the shoot and AM colonization phenotype of loss-of-function mutant plants.

2 | MATERIALS AND METHODS

2.1 | Plant growth conditions and harvest

The *M. truncatula* plants grown for RNA-sequencing, gene expression, protein localisation and loss-of-function phenotyping were all grown in similar conditions; briefly, seeds of *M. truncatula* ecotypes A17 or R108 (loss-of-function studies only) were surface-sterilized, surface-scarified lightly with sandpaper, imbibed and germinated on filter paper as previously described (S.J. Watts-Williams et al., 2017).

Pre-germinated seedlings were moved into pots inoculated with the AM fungus *R. irregularis* WFVAM10, or mock-inoculated. The growth substrate was a mix of autoclaved fine sand mixed in a ratio of 9:1 with sieved and autoclaved low nutrient soil from the Mallala region of South Australia. The final soil/sand mix had a plant-available (DTPA-extractable) Zn concentration of 0.3 mg Zn kg^{-1}. The *R. irregularis* inoculum comprised dry soil, root pieces, spores and hyphae from a pot culture where *R. irregularis* was previously cultured on Marigold (*Tagetes patula*) seedlings for 12 weeks. The mock inoculum was cultured in the same way but without the addition of *R. irregularis* to the culture. For each pot, 630 g of the sand/soil growth substrate was mixed with 70 g of the AM fungal or mock inoculant substrate before transplantation. Plants were grown in a controlled environment chamber with day/night conditions set at 24°C/20°C and 16 h/8 h of light/dark. Plants were watered until draining with reverse osmosis (RO) water three times per week. To ensure the only limiting plant essential nutrient was Zn, plants were given 10 mL each of a modified Long-Ashton solution with Zn omitted from the micronutrient cocktail, twice during the growing period.

For the RNA-sequencing there were three soil Zn treatments: no Zn addition, 5 and 20 mg Zn kg^{-1}. The no Zn addition treatment is considered Zn deficient for *M. truncatula* plants (Peverill et al., 1999). The Zn addition treatments were chosen based on S.J. Watts-Williams et al. (2017), whereby at 5 mg Zn kg^{-1} biomass was the same in the mock- and AM-inoculated plants, while at 20 mg added Zn kg^{-1}

the mock-inoculated plants were negatively affected by the high soil Zn concentration compared to the AM-inoculated plants, indicating Zn toxicity. Plants were destructively harvested after 33 days, and roots washed clean with RO water were snap frozen in liquid nitrogen before storage at −80°C.

For the other plant growth experiments, plants were destructively harvested after 35 days. Shoots were cut at the soil level and roots were washed clean before a subsample of fresh root biomass was moved into 70% ethanol for determination of AM colonization. The shoots and remaining root material were dried at 60°C for at least 48 h before dry weights were determined. Following that, the entire shoot material was homogenized and digested in 4:1 nitric acid:hydrogen peroxide at 125°C for 3 h before being diluted with RO water and analysed for elemental concentrations of P, Mg, K, Zn, Mn and Fe by ICP-OES. The fresh root subsamples were rinsed well and moved into a 10% potassium hydroxide (KOH) solution at room temperature for 7 days to clear the root cells. Cleared roots were rinsed well then stained in a 5% ink in vinegar solution (Vierheilig et al., 1998) at 60°C for 10 min before being stored in 50% glycerol. Colonization by *R. irregularis* was determined on the stained roots following McGonigle et al. (1990) whereby arbuscular, vesicular and hyphal root length colonized were each independently estimated on 100 roots intersects per sample.

2.2 | RNA sequencing

2.2.1 | RNA isolation and sequencing

For all experiments a subsample (~100 mg) flash frozen root material was homogenized in 2 mL microcentrifuge tubes with two 2.8 mm ceramic beads per tube, in a bead beater for 2 × 30 s (Genogrinder). Total RNA was subsequently isolated using a Plant Total RNA kit (Sigma) including on-column DNase treatment following the manufacturer's instructions. The quality and yield of the resulting RNA was analysed using a BioAnalyzer instrument (for RNA-sequencing) or Nandrop (for qRT-PCR). Three biological replicates of each treatment were used in the library preparation for RNA sequencing. RNA-seq libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit according to the manufacturer's instructions (Illumina) and sequenced on a NextSeq. 550 system (Illumina) as 75 bp single-end reads with an average quality score (Q30) of above 92%. RNA-seq data was deposited at the NCBI Sequence Read Archive (NCBI SRA) under project ID PRJNA660297.

2.2.2 | Bioinformatics and analysis of differentially expressed genes (DEGs)

Quality control of RNA-seq data was performed using the FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Transcript abundances as transcripts per million and estimated counts were quantified on a gene level by pseudo-aligning reads against a k-mer index build from the representative transcript models downloaded for the *M. truncatula* Mt4.0 annotation (Tang et al., 2014) using a k-mer length of 31 using the kallisto programme with 100 bootstraps (Bray et al., 2016). Only genes with at least five counts were included in the further analysis. The programme sleuth with a Wald test was used to test for differential gene expression (Pimentel et al., 2017). DEGs were calculated as the log fold change (FC) of the mean *R. irregularis*-inoculated plants to the mock-inoculated plants, for each soil Zn treatment, respectively. Genes were considered as differentially expressed with a $|\log_2(FC)| > 1.9$ and false discovery rate <0.05.

For further analyses, hierarchical clustering and generation of heat maps the Partek Genomics software suite version 6.16 (Partek Incorporated, http://www.partek.com/) was used. Venn diagrams were constructed (http://bioinformatics.psb.ugent.be/webtools/Venn/) to visualize the separation of DEGs into the three Zn treatments or combinations thereof. Gene ontology (GO) term singular enrichment analysis and visualization by hierarchical tree graphs of the AM-induced DEGs was completed using the agriGO tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php) (Tian et al., 2017). Bubble plot of enriched GO terms was plotted using SRplot (https://www.bioinformatics.com.cn/en).

2.3 | Characterization of *MtZIP14*

2.3.1 | Expression of MtZIP14 in roots colonized by AM fungi

To confirm the expression pattern of *MtZIP14* in AM-inoculated compared with mock-inoculated plants, qRT-PCR was performed on material from an independently conducted experiment (see Nguyen et al., 2019). Briefly, *M. truncatula* A17 was inoculated with *R. irregularis* or mock-inoculated and grown in a soil with one of four different soil Zn concentrations; there were eight biological replicates. Total root RNA was isolated as described above and expression of *MtZIP14* was measured by qRT-PCR and normalized to the geometric mean of two housekeeping genes (*MtEF1α* and *MtASPP*) following (Vandesompele et al., 2002) (Supporting Information: Table S9).

2.3.2 | Complementation of a Zn-deficient yeast strain with MtZIP14

The complete mRNA sequence of MtZIP14 cv. A17 from ATG to stop codon was amplified using Phusion High-fidelity DNA polymerase, with the Gateway-specific sequence (CACC) added to the 5′ end of the forward primer. The resulting product was cloned into the pENTR-D-TOPO Gateway-compatible entry vector, transformed by heat shock into *E. coli* DH5α competent cells, and sequenced to confirm before LR reaction to recombine into the yeast expression

vector pDEST52. Successful recombination was confirmed by enzyme digestion. Then, the pDEST52:MtZIP14 construct and empty pDEST52 vector were respectively transformed into the yeast strain ZHY3 (*zrt1zrt2* mutated) and the wild-type (WT) yeast strain DY1457 (both kindly provided by Prof. D. Eide) using the lithium-acetate transformation method (Gietz & Woods, 2002). Transformants were selected on yeast nitrogen base (YNB) minus uracil plus 2% glucose plates.

For the yeast growth studies, the ZHY3 yeast strain expressing the empty vector or MtZIP14 construct were grown overnight in liquid YNB −uracil with 2% galactose. Cells were pelleted, washed three times in sterile water and resuspended in YNB −uracil with 2% galactose media supplemented with 1 mM EDTA and one of 0.1, 0.2, 0.5, 1.0 or 1.5 mM Zn as $ZnSO_4$ to an OD of ~0.23. The EDTA was added for the purpose of chelating the existing Zn in the media (400 µg Zn L^{-1} according to manufacturer) and allowed for the creation of media completely devoid of Zn (following López-Millán et al., 2004). The availability of free Zn^{2+} in the EDTA-YNB media was predicted using the Visual MINTEQ software (https://vminteq.lwr.kth.se/). Without any addition of $ZnSO_4$, the EDTA-YNB media was predicted to have approximately 0.001 nM free Zn^{2+}, and the addition of 0.1 mM $ZnSO_4$ yielded 0.048 nM free Zn^{2+}. At the highest $ZnSO_4$ addition of 1.5 mM, the EDTA-YNB media had a predicted free Zn^{2+} availability of 237.86 µM.

A 150 µL aliquot of yeast cells was placed into a 96-well microplate for each treatment (three replicates of each) and the plate sealed with sterile film. The microplate was placed into a reader (BMG Omega) and growth of the yeast strains was quantified over 66 h. Solid agar plates were prepared from YNB −uracil with 2% galactose and 1 mM EDTA with the addition of Zn at 0, 0.2, 0.5 or 1 mM $ZnSO_4$. The WT and ZHY3 yeast empty vector constructs and the ZHY3-MtZIP14 construct were cultured overnight in 5 mL of YNB −uracil with 2% galactose. The resulting cultures were rinsed well with sterile water three times before being resuspended in 3 mL sterile water and diluted to an OD_{600} of 0.5, 0.1, 0.01 and 0.001. For each yeast construct, 5 µL of each dilution was spotted onto the prepared EDTA-YNB agar plates, and onto control YNB −uracil with 2% galactose or 2% glucose (no EDTA) plates and placed inverted in a 28°C incubator for 2–4 days. The experimental plates were replicated three times. The plates with WT yeast harbouring the empty vector construct were photographed after 2 days and the ZHY3 yeast harbouring the empty vector or MtZIP14 constructs after 4 days, due to faster growth of the WT strain.

The ZHY3 yeast empty vector construct and the ZHY3-MtZIP14 construct were cultured in liquid YNB −uracil with 0.2 mM $ZnSO_4$ and 1.0 mM EDTA added ($n = 3$). After 48 h, the yeast biomass was pelleted, washed with sterile water, pelleted again, supernatant removed and biomass weighed before the cells were digested in 400 µL 36% nitric acid at 95°C until dry. Dry cells were reconstituted in 2% nitric acid, sonicated and filtered before being analysed for Zn concentration by ICP-MS.

2.3.3 | Phenotyping of loss-of-function *MtZIP14* mutant plants

To find the *MtZIP14* gene sequence in the *M. truncatula* R108 ecotype, the *MtZIP14* A17 ecotype mRNA sequence was compared using a BLAST online tool (http://www.medicagohapmap.org/tools/r108_blastform).

Line numbers NF8057 and NF4665 from the Noble Foundation's *M. truncatula* Tnt1 insertion mutant collection were predicted to have an insertion in the *MtZIP14* gene as per BLAST analysis of R108 sequence in the Tnt1 insertion collection database (https://medicago-mutant.noble.org/mutant/database.php). The NF8057 line has a Tnt1 insertion in the first exon of the *MtZIP14* gene sequence, approximately 250 nucleotides downstream of the ATG. The NF4665 line has a Tnt1 insertion in the first exon approximately 496 nucleotides downstream of the ATG. Genotyping of the R1 plants supplied by the Noble Foundation using gene-specific and Tnt1-specific primers identified a number of plants homozygous for the Tnt1 insertion, which were subsequently genetically backcrossed using the keel petal incision method to the R108 WT background (following Veerappan et al., 2014). The resulting heterozygous progeny were grown and allowed to self-pollinate, then homozygous and out-segregated WT progeny were isolated for use in subsequent experiments. One full back-crossing and reisolation event was conducted before the phenotyping experiments.

To investigate the *mtzip14* phenotype, *M. truncatula* R108 *mtzip14*, the respective out-segregated WT for each NF line, and R108 WT plants were inoculated with *R. irregularis* or with mock inoculum and grown in a Zn-deficient soil, as described above. Plants were harvested 35 days after transplantation; measurements of dry shoot and root biomass, AM colonization (arbuscular, vesicular, hyphal) and shoot Zn concentration were taken from distinct plant samples. Flash frozen root samples were taken from one experiment for the isolation of RNA and gene expression analysis by qRT-PCR (oligonucleotide primer sequences in Supporting Information: Table S9). A biological replicate was considered one plant in a pot. Each treatment had six biological replicates in each experiment (60 plants total); the phenotyping experiment was conducted in an identical manner, independently, three times (180 plants total).

An additional phenotyping experiment was set up to quantify the effects of different soil Zn concentrations (0, 5 and 20 mg Zn kg^{-1} soil) on the NF8057 *mtzip14* plants. The soil/sand mix and AM fungal inoculation methods followed previous phenotyping experiments reported here, and a small amount of P (2 mg P kg^{-1} soil) was mixed throughout the soil so that any potential interactions with P deficiency were avoided. The unamended sand/soil mix is referred to as Zn0 hereafter; 5 mg or 20 mg Zn kg^{-1} soil was added to establish the Zn5 and Zn20 treatments, respectively. The plants were also given 20 mg N throughout the growing period to mitigate N deficiency. The plants were grown for 37 days in a controlled environment room (conditions as described above) before being destructively harvested. Each experiment had five biological replicates. Measurements of shoot biomass, shoot Zn concentration

(by ICP-OES) and AM colonization of roots were taken, as described above.

2.4 | Statistical analysis and data presentation

A linear mixed effects model (two-tailed) was employed to analyze the shoot biomass, shoot Zn, P, Mg, K, Fe and Mn concentrations, and AM colonization data using the 'lme' function within the 'nlme' package in R version 4.0.2 (R Core Team, 2022). *Mycorrhiza* and *Genotype* (and their interaction) were included as fixed effects and a random term for *Experiment* was included to block the data by the experiment it originated from. This allowed for data from the three replicated phenotyping experiments to be included in the model while accounting for effects of the individual *Experiment*. The NF4665 and NF8057 Tnt1 lines were statistically analysed separately. For the NF8057 *ZIP* gene expression data, a two-way analysis of variance (ANOVA; two-tailed) was employed with *Mycorrhiza* and *Genotype* as the factors. Plant data from the Zn addition experiment was analysed by two-way ANOVA with *Mycorrhiza* and *Genotype* the factors, for each soil Zn treatment, respectively. Where the interaction or main effects were significant ($p < 0.05$), the 'lsmeans' package and function were used to conduct Tukey's HSD post hoc pairwise comparisons between the treatments and identify any significant differences. These are presented as letters on the relevant figures.

The Tnt1 plant physiological and gene expression data are presented as box-and-whisker plots (one representative experiment presented in main figures), and were generated using the 'ggboxplot' function within the 'ggpubr' package with 'jitter' added to visualize the individual data points and outliers. A principal components analysis (PCA) was undertaken using the 'PCA' function in the 'FactoMineR' package, including all of the available plant biomass, AM colonization and nutrient content data to visualize the effect of *Mycorrhiza* and *Genotype* on the data. The PCA biplot was drawn using the 'factoextra' package; the group mean was also computed for each level, and a 95% confidence ellipse drawn around the mean to determine significant differences between groups.

3 | RESULTS

3.1 | Identification of Zn-related genes linked to AM colonization in a root transcriptome

To identify genes potentially involved in the AM fungal uptake of Zn a RNA-seq experiment was performed using *M. truncatula* grown at different soil Zn concentrations and inoculated with the AM fungus *R. irregularis* compared to mock inoculation (Supporting Information: Table S1).

In order of increasing soil Zn addition (0, 5, 20 mg Zn kg^{-1}), there were 589, 201 and 918 genes that were significantly up-regulated with AM colonization (Figure 1a) and 221, 33 and 159 genes that were down-regulated (Figure 1b). There were 165 genes that were significantly up-regulated by AM colonization common to all three soil Zn treatments (list in Supporting Information: Table S2), which included the phosphate transporter gene *MtPT4* (Harrison et al., 2002), the aquaporin *MtNIP1* (Uehlein et al., 2007) and two ABC subfamily B transporter genes (Roy et al., 2021). This list of common DEGs was used to generate a GO term enrichment plot that highlighted the biological processes and molecular functions related to the up-regulated genes (Figure 1c). The most highly enriched GO term was 'cysteine-type peptidase activity' (not shown in figure due to *x*-axis scale) at 8.8% enrichment. Other enriched GO terms included transporter activity and oxidoreductase activity.

The DEGs in each Zn treatment were considered separately; at Zn0, the genes up-regulated by AM colonization were associated with the enriched GO terms copper, iron and manganese ion binding (Supporting Information: Figure S1a; Table S3). There were also multiple GO terms related to oxidoreductase and hydrolase activity that were enriched in the Zn0 DEG list. At Zn5, the GO terms associated with heavy metal binding were no longer represented in the DEGs, while oxidoreductase and hydrolase activity remained (Supporting Information: Figure S1b; Table S4). At Zn20, oxidoreductase activity was highly enriched in DEGs responding to AM colonization, as were endopeptidase inhibitor activity and copper and iron ion binding (Supporting Information: Figure S1c; Table S5).

The mock-inoculated plants displayed marked changes in transcript abundance with Zn-deficiency (Zn0 compared to Zn5) (135 up, 538 down); GO terms that were enriched in the up-regulated DEG list included oxidoreductase activity and iron ion binding (molecular functions; Supporting Information: Figure S1d) and oxidation reduction (biological processes; Supporting Information: Figure S1e). In comparison, the AM-colonized plants had few differentially regulated genes in response to Zn deficiency (29 up, 37 down), and no significantly enriched GO terms related to those two lists of DEGs.

Three lists of candidate genes with a potential role in AM fungal Zn nutrition were compiled based on their gene annotation as Zn transporter (ZIP), heavy metal transporter or Zn-binding (Supporting Information: Tables S6–8). Of all 16 annotated *ZIP* genes in *M. truncatula*, only one (*MtZIP14*; *Medtr6g007687*) was exclusively expressed in AM colonized root cells (Supporting Information: Figure S2a), and another gene was up-regulated in all Zn treatments (*MtZIP7*; *Medtr3g058630*), while no others showed AM specificity (Figure 2a). A previous study showed that the MtZIP7 is a manganese (Mn) transporter (López-Millán et al., 2004), and suggested that MtZIP7 is not primarily involved in Zn uptake. The only remaining and most promising candidate, *MtZIP14*, was uncharacterized, and showed an expression pattern consistent with involvement with AM fungal colonization. Quantitative RT-PCR on samples from an independent experiment confirmed that *MtZIP14* is almost exclusively expressed in plants that have been colonized by *R. irregularis* (Figure 2b). Expression of *MtZIP14* was not affected by increasing soil Zn concentration (0, 5, 10, 20 mg kg^{-1} added Zn), and there was a positive ($R^2 = 0.708$), significant ($p < 0.0001$) relationship between

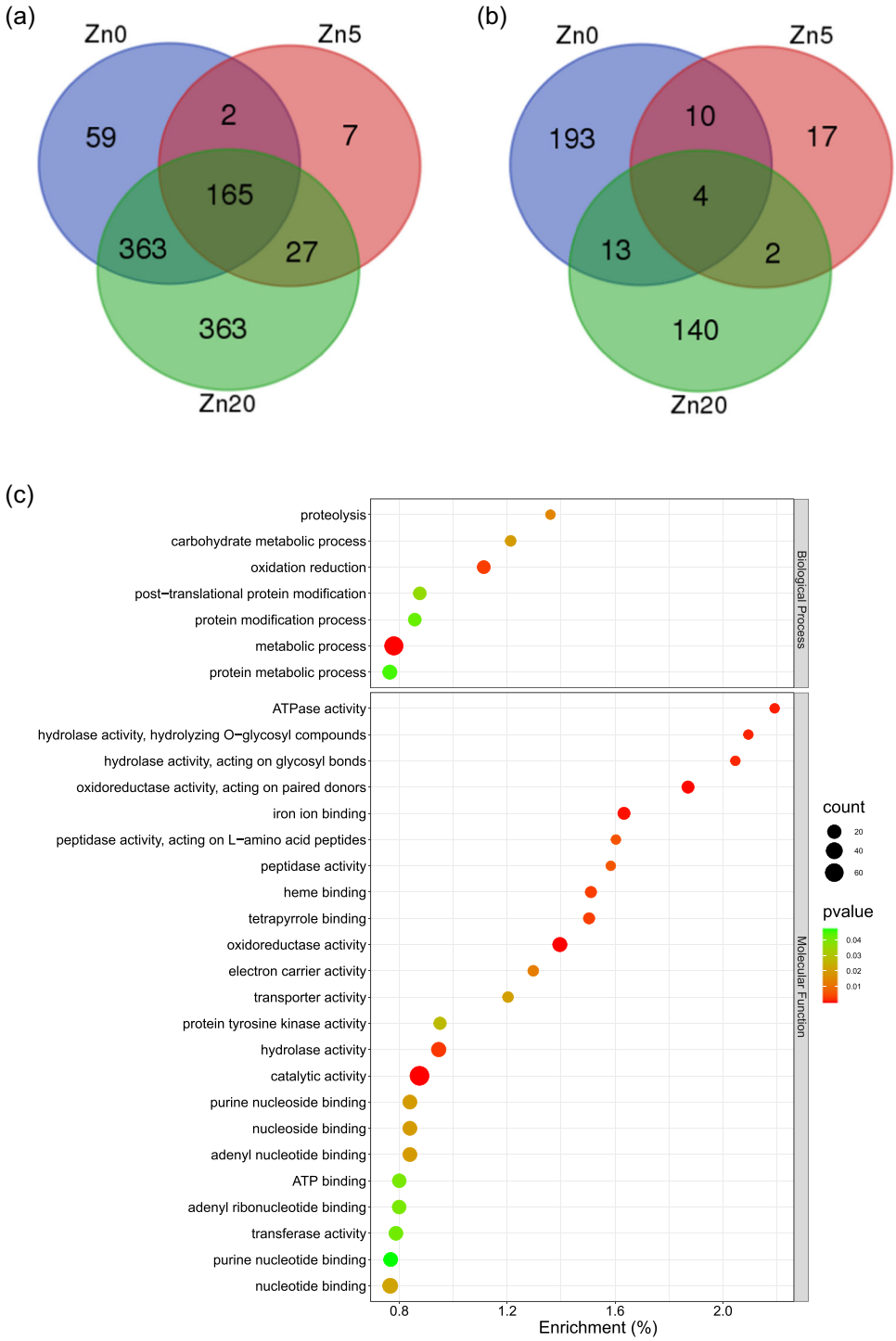

FIGURE 1 Numbers of significantly up-regulated (a) and down-regulated (b) *Medicago truncatula* A17 genes by *Rhizophagus irregularis* colonization, split into three soil Zn addition treatments: Zn0 no addition; Zn5 5 mg Zn kg^{-1} addition; Zn20 20 mg Zn kg^{-1} addition, $n = 3$. Bubble plot illustrating enriched GO terms from a list of genes significantly up-regulated by AM colonization across all three soil Zn treatments, split into biological processes (top) and molecular functions (bottom). AM, arbuscular mycorrhizal; Zn, zinc.

expression of *MtZIP14* and the AM fungal marker gene, *R. irregularis α-tubulin*, suggesting that increased colonization by the AM fungus is associated with increased expression of *MtZIP14* (Figure 2c).

Two genes encoding HMA-domain proteins contained in the heavy metal transporter list were up-regulated by AM colonization: one in Zn 0 and 5 (*Medtr0041s0140*) and one in all Zn treatments (*Medtr6g051680*) (Supporting Information: Figure S2b). HMA-domain proteins play key roles in transporting monovalent and divalent ions in plants, and in detoxification (Shi et al., 2019). In the Zn-binding candidate list there was a Zn-binding dehydrogenase oxidoreductase gene up-regulated

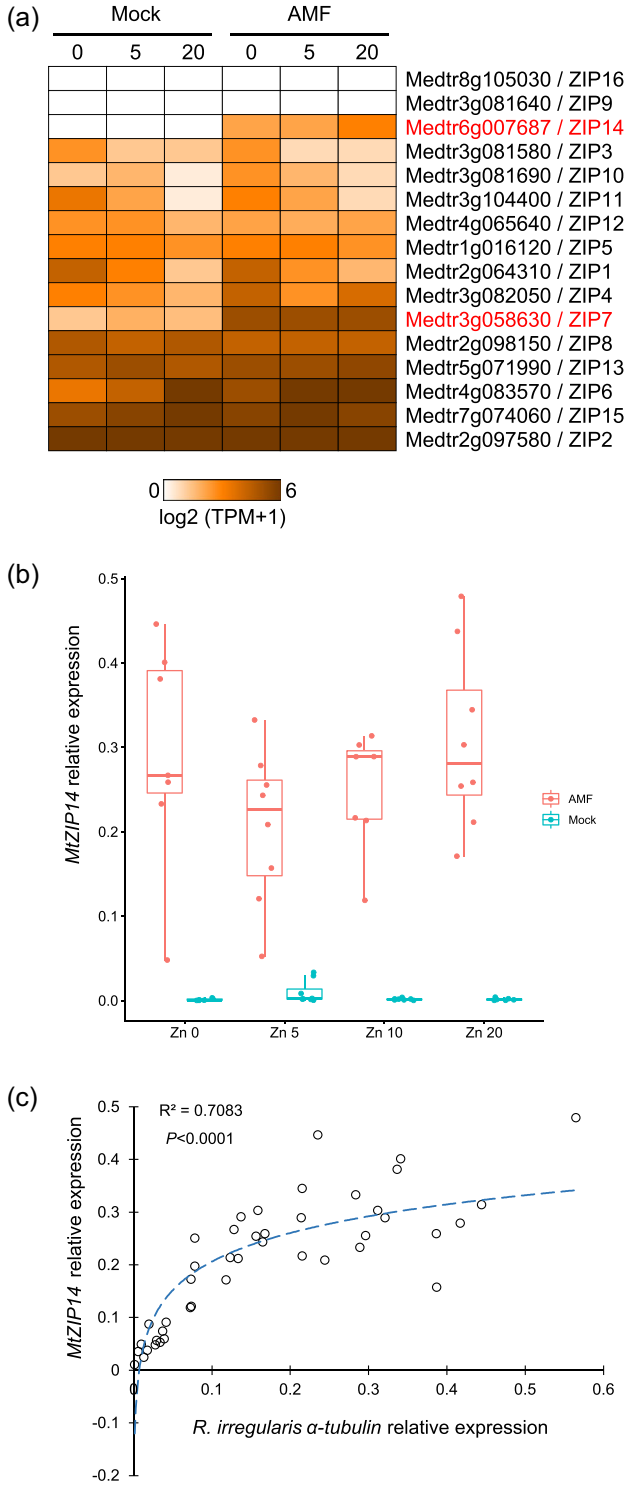

FIGURE 2 Expression of 16 *Medicago truncatula* genes annotated as ZIP transporters (a) split into three soil Zn addition treatments: Zn0 no addition; Zn5 5 mg kg^{-1} addition; Zn20 20 mg kg^{-1} addition. Genes highly up-regulated in AM colonized plants across all three Zn addition treatments are highlighted in red. Expression of *MtZIP14* in the roots of *Rhizophagus irregularis*-inoculated (pink) and mock-inoculated (blue) plants grown at four soil Zn additions: Zn0 no addition; Zn5 5 mg Zn kg^{-1} addition; Zn10 10 mg Zn kg^{-1} addition; Zn20 20 mg Zn kg^{-1} addition, $n = 8$ (b) and relationship between *R. irregularis α-tubulin* and *MtZIP14* gene expression with a logarithmic trendline (c). Gene expression was calculated as the gene-of-interest relative to the geometric mean of two housekeeping genes, *MtEF1α* and *MtASPP* (Supporting Information: Table S9). AM, arbuscular mycorrhizal; Zn, zinc. [Color figure can be viewed at wileyonlinelibrary.com]

in all Zn treatments (*Medtr8g035880*) (Supporting Information: Figure S2c); Zn-binding alcohol dehydrogenases catalyse the reduction of acetaldehyde to ethanol, mainly in meristematic tissues such as root apices under anaerobic conditions (Marschner, 2012). The expression of these three genes were determined in an independent experiment using quantitative RT-PCR; the HMA-domain protein *Medtr6g051680* was induced by AM colonization across all Zn conditions (Supporting Information: Figure S3a), while *Medtr0041s0140* was down-regulated by AM colonization in this experiment (Supporting Information: Figure S3b). The Zn-binding dehydrogenase oxidoreductase gene (*Medtr8g035880*) was exclusively expressed in AM colonized roots in all the soil Zn treatments (Supporting Information: Figure S3c).

MtZIP14 was the most highly up-regulated of the *ZIP* genes in roots with AM colonization. In silico analysis predicted a potential metal-binding domain rich in histidine residues between trans-membrane III and IV, similar to the other ZIP proteins with Zn-transport function characterized in *M. truncatula* [MtZIP1, 2, 5, 6; (López-Millán et al., 2004; Stephens et al., 2011)] (see protein sequence alignment Supporting Information: Figure S4).

3.2 | MtZIP14 in the *zrt1zrt2* yeast expression system

To test for the involvement of MtZIP14 in Zn transport, *MtZIP14* was cloned into a heterologous expression system—yeast (*Saccharomyces cerevisiae*) lacking its two plasma membrane Zn transporters; the yeast strain ZHY3 [*zrt1zrt2*; (Zhao & Eide, 1996a, 1996b)] displays reduced growth under low Zn conditions. In the EDTA-only YNB media, neither the empty vector control nor the MtZIP14 expressing ZHY3 yeast strain grew, confirming the growth defect of ZHY3 (*zrt1zrt2*) mutant (Figure 3a). Interestingly, already in the lowest Zn addition concentration (0.1 mM) where Zn^{2+} was available at nanomolar concentration, the MtZIP14-expressing yeast grew, while the empty vector ZHY3 strain did not (Figure 3b), suggesting that MtZIP14 is able to mediate Zn uptake from very low external Zn concentrations and is likely a high affinity Zn transporter. In the 0.2 and 0.5 mM added Zn EDTA-YNB (Figure 3c,d), the empty vector displayed slow growth with OD_{600} increasing after 35 h, suggesting that higher Zn is sufficient to enable this yeast strain to survive. Expression of MtZIP14 significantly increased yeast growth over the empty vector control, which was especially evident at 0.5 mM Zn, where MtZIP14 growth peaked (Figure 3g). However, growth of the MtZIP14-expressing yeast was reduced at 1 mM added Zn compared to the empty vector control, suggesting the transport of Zn via MtZIP14 resulted in Zn influx to toxic concentrations and inhibited

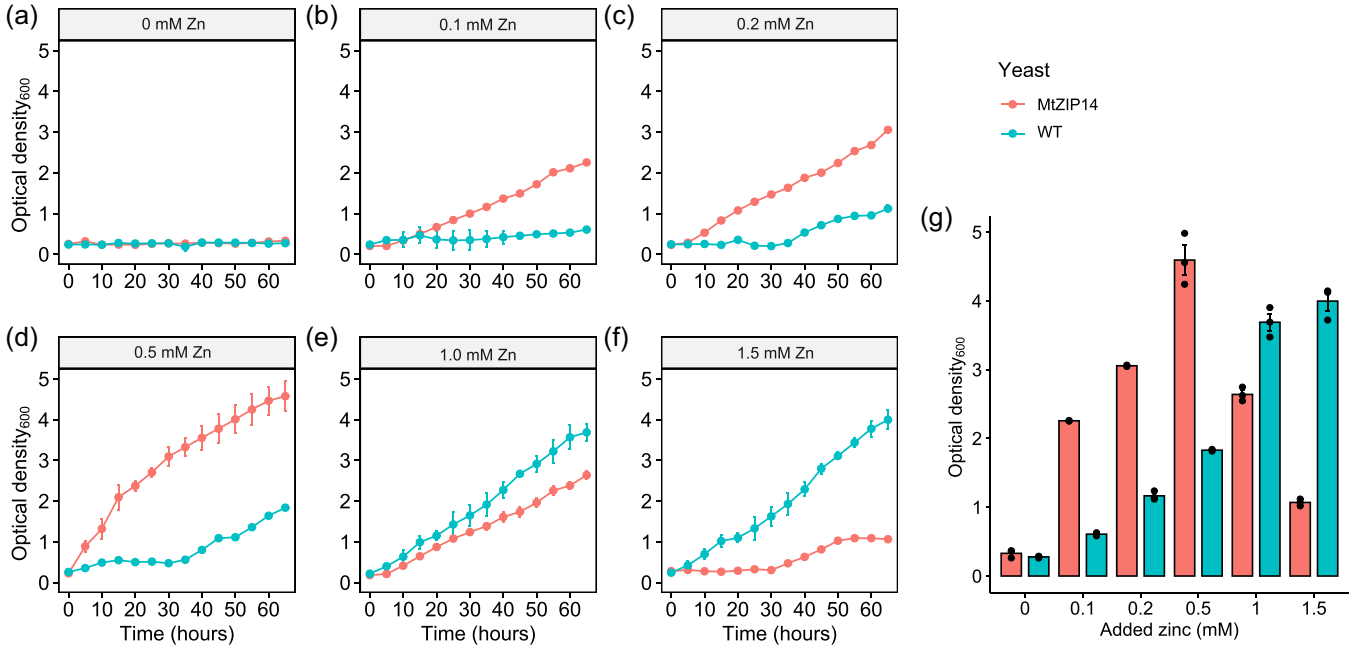

FIGURE 3 Complementation of the Zn transporter (*zrt1zrt2*) mutant (ZHY3) yeast strain with MtZIP14 (pink), or with the empty vector pDEST52 (blue), grown over a 66 h period in liquid YNB −uracil media with 2% galactose and 1 mM EDTA. With the addition of EDTA only (a), there was no growth of any yeast strains without Zn supplementation. The Zn supplementation treatments were 0.1 (b), 0.2 (c), 0.5 (d), 1.0 (e) and 1.5 (f) mM $ZnSO_4$. After 66 h of growth the final OD_{600} all treatments was recorded (g). Values are mean ± standard deviation of the mean, $n = 3$. YNB, yeast nitrogen base; Zn, zinc. [Color figure can be viewed at wileyonlinelibrary.com]

growth of the yeast (Figure 3e). At the highest Zn addition (1.5 mM), the empty vector yeast grew well but the MtZIP14-expressing yeast did not grow until 35 h, and growth thereafter was poor, providing further support for the toxicity hypothesis (Figure 3f). Growth of ZHY3 on the solid YNB agar media for 96 h followed the same pattern as the liquid YNB; MtZIP14-expressing yeast grew in all Zn treatments and best at the 0.5 mM added Zn (Supporting Information: Figure S4a–c), while the empty vector yeast grew well only at 1 mM added Zn. The WT positive control yeast strain (DY1457) grew on all solid agar experimental conditions with Zn addition (Supporting Information: Figure S5a–c). The mean concentration of Zn in ZHY3-MtZIP14 cells grown at 0.2 mM Zn^+ 1.0 mM EDTA was 1.503 ± 0.363 µM, which was greater than in the ZHY3-pDEST52 (empty vector) cells: 0.854 ± 0.234 µM (Student's *t*-test *p*-value of 0.026).

3.3 | Phenotype of *mtzip14* loss-of-function mutant plants

M. truncatula plants with Tnt1 retrotransposon insertion in the *MtZIP14* gene were isolated and analyzed. The generated *mtzip14* plants had either no detectable expression of *MtZIP14* (NF8057) or a strongly reduced expression (NF4665) to approximately one-third of the out-segregated WT (Supporting Information: Figure S6). In NF8057 the retrotransposon insertion is in the second predicted transmembrane helix of MtZIP14, and in NF4665 it is in a hydrophilic loop between helix three and helix four. Based on these predictions, both lines can be considered as effectively loss-of-function. As a control, out-segregated WT plants from those two lines were used; the roots of the WT plants expressed *MtZIP14* when colonized by the AM fungus *R. irregularis*, while the mock-inoculated plants had no *MtZIP14* expression, confirming the results obtained with WT plants in the RNA-seq experiment.

The shoot biomass of both *mtzip14* plant lines was reduced when compared to the out-segregated WT plants when colonized by *R. irregularis* under Zn deficient conditions (Figure 4a; Supporting Information: Table S11; Table S12), with no significant difference for the mock-inoculated plants. Shoot Zn concentrations (μg^{-1}) increased in AM-colonized plants (Figure 4c); meanwhile, colonized *mtzip14* plants had lower Zn content (µg Zn $plant^{-1}$) than the WT plants, whereas, the mock-inoculated *mtzip14* and WT plants contained similar amounts of Zn (Figure 4b). Shoot P concentrations and P contents increased in AM-colonized plants compared to mock-inoculated plants regardless of *Genotype* (Supporting Information: Figure S7a, S8a), while shoot Mg concentrations and contents showed the opposite trend, being greater in the mock-inoculated plants (Supporting Information: Figure S7c, S8c). In the NF8057 line only, shoot K contents (Supporting Information: Figure S8b) and shoot Mn contents (Supporting Information: Figure S8e) displayed a similar trend to that of Zn contents, whereby the AM-colonized *mtzip14* plants had lower values than the WT plants. The Zn addition phenotyping experimental and multivariate analysis were used to further

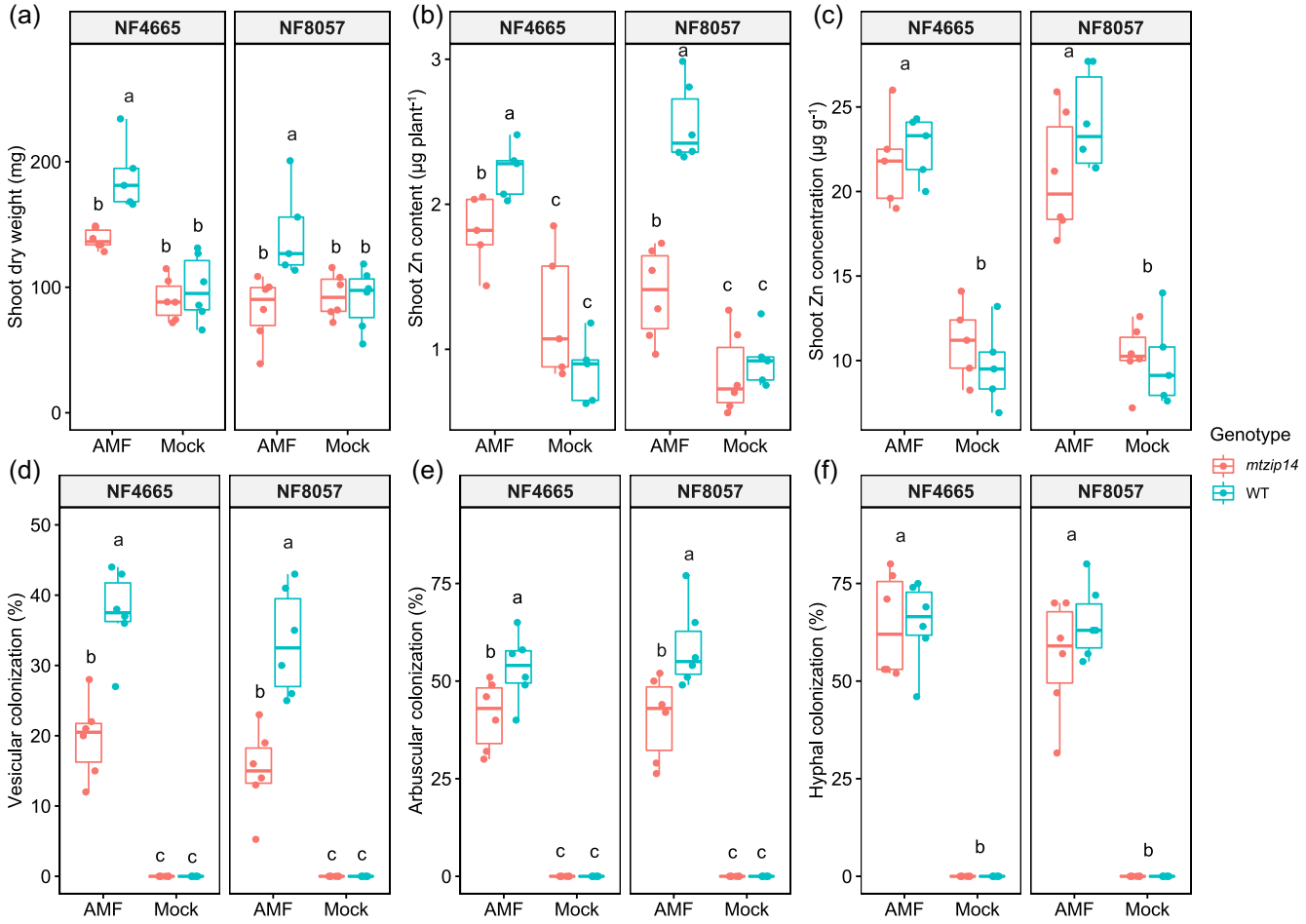

FIGURE 4 Shoot dry weight (mg) (a), shoot Zn content (µg Zn plant^{-1}) (b), shoot Zn concentration (µg Zn g^{-1}) (c), root length colonized by AM fungus *Rhizophagus irregularis* in terms of percentage vesicles (d), arbuscules (e) and internal hyphae (f) in the *mtzip14* (pink) and segregating WT (blue) lines grown with or without inoculation by the AM fungus *R. irregularis*, $n = 6$. Means with different letters are considered significantly different ($p < 0.05$) as per Tukey's HSD post hoc test. Where one letter appears above two boxes, it represents a significant main effect of *Mycorrhiza* where the two genotypes are pooled. AM, arbuscular mycorrhizal; WT, wild-type; Zn, zinc. [Color figure can be viewed at wileyonlinelibrary.com]

explore a potential link between *MtZIP14* function and Zn nutrition of AM-colonized plants.

For both *mtzip14* lines, vesicular (Figure 4d) and arbuscular (Figure 4e) colonization were both lower than the WT, while hyphal colonization of roots was not significantly different (Figure 4f). Closer examination of the internal fungal structures in the WT and *mtzip14* genotypes under the stereomicroscope did not reveal any morphological differences in mature arbuscules (Supporting Information: Figure S9a–c). There was no colonization by AM fungi in the mock-inoculated plants; and shoot biomass, Zn nutrition and root AM colonization were comparable in the R108 WT plants and segregated WT lines (Supporting Information: Figure S10a–f).

How the loss of *MtZIP14* function altered the expression of other *ZIP* transporter genes was investigated by analysing 15 additional *MtZIP*s by quantitative RT-PCR from the NF8057 *mtzip14* and WT roots. Majority of the *ZIP* genes measured were highly down-regulated in the AM colonized roots compared to the mock-inoculated roots, regardless of genotype (i.e., both *mtzip14* and WT were similarly down-regulated in AM colonized roots) (Figure 5a–l; Supporting Information: Table S10). As expected, *MtZIP7* was the only *ZIP* gene up-regulated by AM colonization, and it was more highly up-regulated in *mtzip14* than the WT, suggesting a transcriptional impact on *MtZIP7* due to the loss of *MtZIP14* function (Figure 5g). No transcripts were detected for *MtZIP13* or *MtZIP16*.

3.4 | Zn addition phenotyping experiment

Shoot dry weights at Zn0 followed the same treatment pattern as the initial *mtzip14* phenotyping experiments, but were generally greater, owing to the additional P supplied in this experiment (Figure 6a); the WT plants colonized by *R. irregularis* were larger than all the *mtzip14* plants, and WT grown without AM inoculation. The plants were generally smaller at Zn5 but the *Genotype * Mycorrhiza* interaction

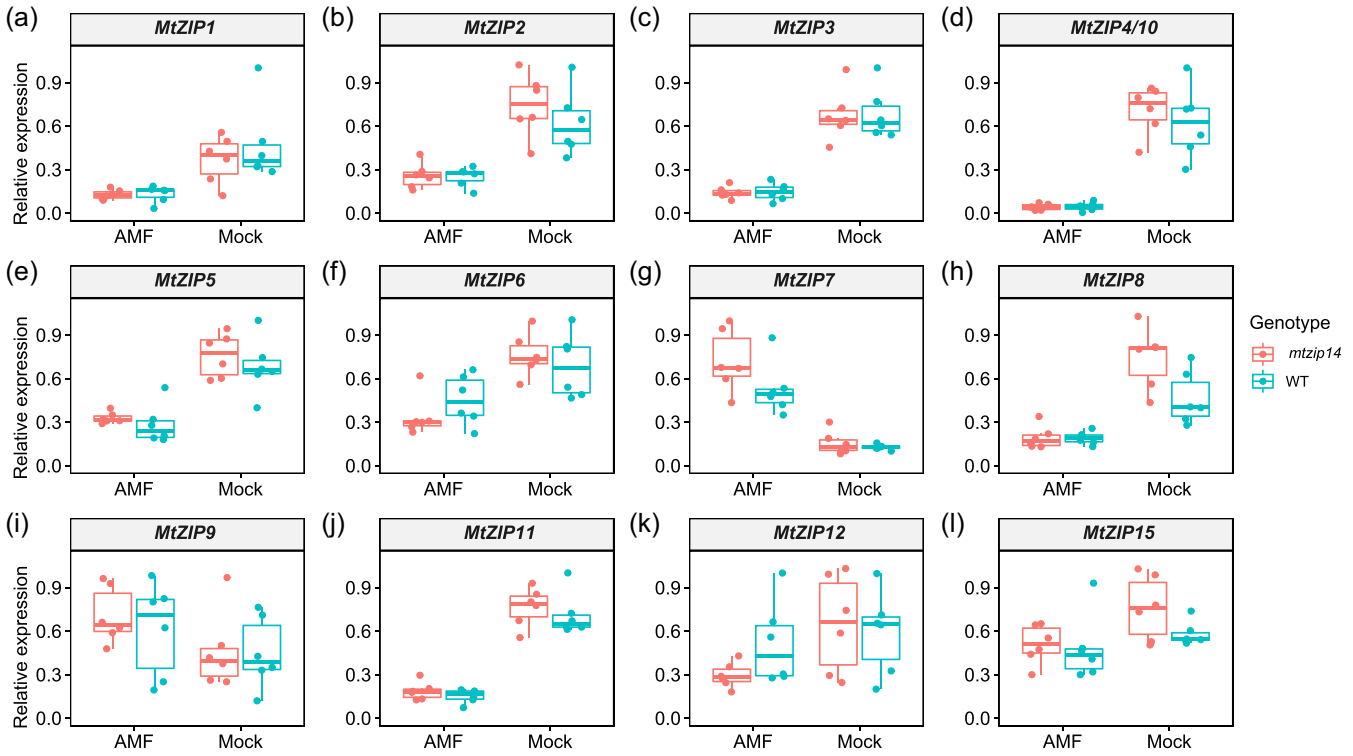

FIGURE 5 Expression of 12 *ZIP* transporter genes (a–j), in the roots of NF8057 *mtzip14* (pink) and segregating WT (blue) genotypes grown with or without inoculation by the AM fungus *Rhizophagus irregularis*. Gene expression was calculated as the gene-of-interest relative to the geometric mean of two housekeeping genes, *MtEF1α* and *MtASPP* (Supporting Information: Table S9), $n = 6$. AM, arbuscular mycorrhizal; WT, wild-type. [Color figure can be viewed at wileyonlinelibrary.com]

was the same as at Zn0 (Supporting Information: Table S13). At Zn20, the plants were smaller again, and there was no difference in shoot biomass due to Genotype or AM inoculation treatment. Shoot Zn contents followed the same pattern as shoot biomass at Zn0 and Zn5, with the AM-colonized WT plants having greater Zn contents than all other treatments (Figure 6b). At Zn20 there was a significant main effect of *Mycorrhiza*, whereby the AM-colonized plants had lower Zn contents than the mock-inoculated plants. Arbuscular, vesicular and hyphal colonization at Zn0 also followed the previous phenotyping experiments (Figure 6d–f), with greater arbuscular and vesicular, but not hyphal, colonization in the WT plants than the *mtzip14* plants. AM colonization generally increased from Zn0 to Zn5, then decreased at Zn20. At Zn5, all of arbuscular, vesicular and hyphal colonization were greater in the WT plants, but at Zn20 only arbuscular and vesicular colonization were greater than in the *mtzip14* plants.

3.5 | Multivariate data analysis

A PCA was conducted to analyze all plant data simultaneously. The PCA loadings showed that AM-colonized WT plants were separated from the AM-colonized *mtzip14* plants by their greater AM colonization (arbuscular, vesicular and hyphal) and shoot Zn contents (Supporting Information: Figure S12).

4 | DISCUSSION

4.1 | A role for MtZIP14 in the AM symbiosis

We have identified MtZIP14 as a plant transport protein that is linked to the function of the AM symbiosis, and appears to be related particularly to plant Zn nutrition. When grown in a Zn-deficient soil, the WT plants with functional *MtZIP14* had a clear benefit over the *mtzip14* plants, and WT shoots produced significantly more biomass. This indicates that the function of *MtZIP14* is critical to the AM-colonized plant being competitive. The advantage of a functional *MtZIP14* when colonized by *R. irregularis* appeared in shoot biomass not only at Zn-deficiency but also when some Zn was added to the soil (Zn5). However, when a high amount of Zn was added to the soil (Zn20), the advantage of *MtZIP14* was nullified. It also suggests that the non-colonized control plants could not compensate for the loss of AM-derived Zn with increased uptake via the direct pathway (i.e., root uptake from the rhizosphere) to reach similar Zn contents as the AM-colonized WT plants. Non-colonized plants took up ~50% of the Zn compared to the colonized WT plants.

Further gene expression analysis of the *mtzip14* genotype revealed that the mock-inoculated plants had higher expression of at least eight *ZIP* genes compared to the AM colonized plants, including four ZIP transporters that have been shown to transport Zn in the *zrt1zrt2* yeast previously (MtZIP1, 2, 5, 6); this suggests that

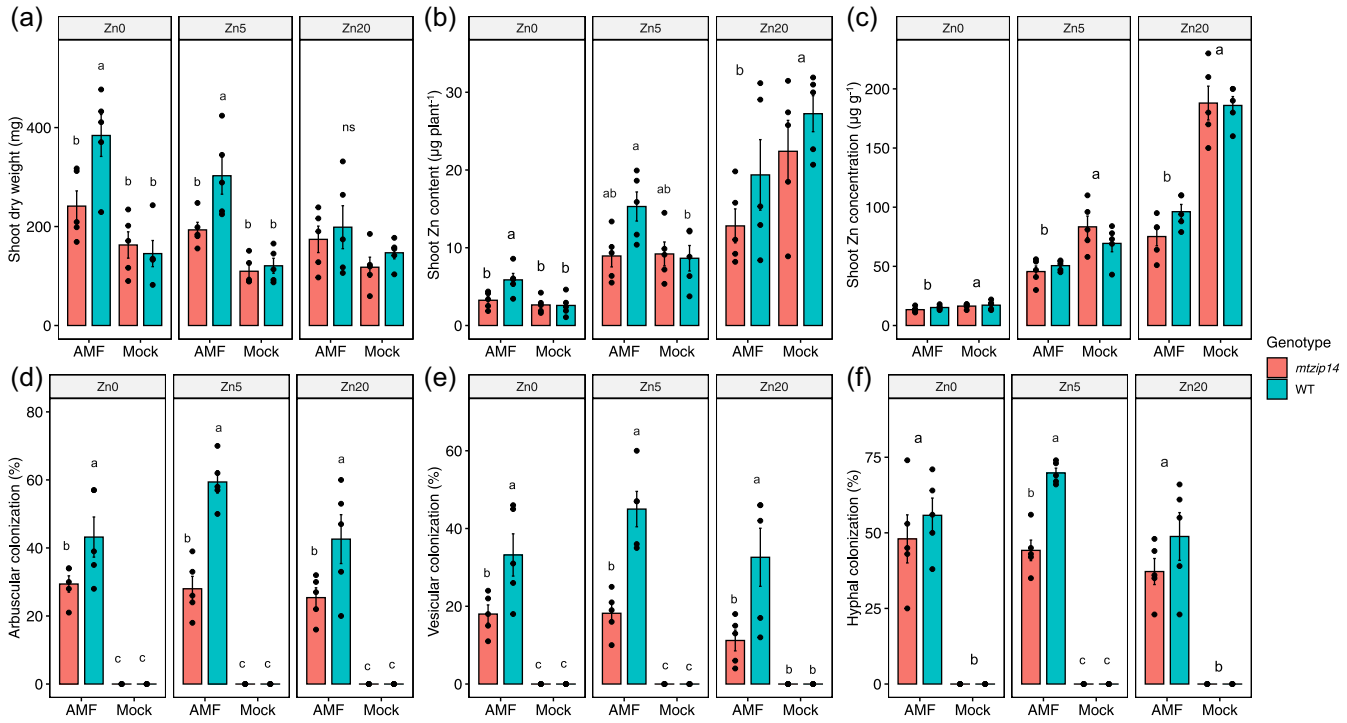

FIGURE 6 Shoot dry weight (mg) (a), shoot Zn content (µg Zn plant^{-1}) (b), shoot Zn concentration (µg Zn g^{-1}) (c), root length colonized by AM fungus *Rhizophagus irregularis* in terms of percentage arbuscules (d), vesicles (e) and internal hyphae (f) in the *mtzip14* (pink) and segregating WT (blue) lines grown with or without inoculation by the AM fungus *R. irregularis*. Values are mean ± standard error of the mean, $n = 5$. Means with different letters are considered significantly different ($p < 0.05$) as per Tukey's HSD post hoc test. Where one letter appears above two bars, it represents a significant main effect of *Mycorrhiza* where the two genotypes are pooled. AM, arbuscular mycorrhizal; WT, wild-type; Zn, zinc. [Color figure can be viewed at wileyonlinelibrary.com]

expression of non-PAM *ZIP* genes are generally suppressed in AM plants. Despite the general down-regulation of *ZIP* transporter genes in the AM-inoculated plants, the Zn concentrations of the AM inoculated plants were still greater. This might suggest that Zn uptake via the AM pathway confers a greater selective advantage to the plant, compared to direct uptake under these conditions.

Without a functional *MtZIP14*, the AM-colonized *mtzip14* plants still accumulated more Zn in their shoots compared to the non-colonized *mtzip14* plants. This indicates that the *mtzip14* plants had a Zn uptake advantage by being colonized by AM fungi, although not to the extent of the WT plants with a functional *MtZIP14*. The source of the advantage may be another AM-specific transporter, besides MtZIP14, that is able to transport Zn across the PAM, which is also expressed in non-colonized plants and was therefore not identified in our RNA-seq analysis. In addition, *MtZIP7* was expressed more highly in the *mtzip14* plants than the WT plants, which may suggest that MtZIP7 might be able to compensate partially for the loss of *MtZIP14* function. MtZIP7 may be able to also transport Zn at a low affinity or low rate, and increased expression and protein abundance might therefore lead to increased Zn uptake in *mtzip14*. Alternatively, the advantage may be due to indirect environmental effects of the AM symbiosis on the availability of Zn in the soil, for example, through exudation that mobilizes Zn in soil, that led to increased plant uptake of Zn via the direct (root) pathway.

Loss of *MtZIP14* function negatively affected the colonization of the roots by *R. irregularis* suggesting a role for the gene in maintaining the plant–fungal association. The proportion of 'functional' AM structures (arbuscules and vesicles) were lower in the *mtzip14* mutant plants, while hyphal colonization was not significantly different, indicating that *MtZIP14* is important for the formation of fungal structures within the root, but not the root colonization event itself. The AM colonization phenotype persisted in the high soil Zn treatment, even though shoot biomass phenotype disappeared.

A similar AM colonization phenotype has been reported in plants lacking the AM-specific Pi transporter gene (*MtPT4*) (Javot et al., 2007), and rice plants lacking a symbiotic nitrate transporter gene (*OsNPF4.5*) (Wang et al., 2020). This suggests that the plant–fungal symbiosis is somewhat disrupted by the loss of *MtZIP14* expression, and that the active sites of nutrient exchange (arbuscules), as well as fungal resource storage units (vesicles), were not produced by the fungus to the same extent due to this disruption. However, the mature arbuscules in *mtzip14* plants did not appear to have any morphological differences compared to those of the WT plants, which contrasts with the phenotype observed in *mtpt4* mutant plants grown under high nitrogen conditions (Javot et al., 2011), and is deserving of further investigation.

The expression of *MtZIP14* was not down-regulated in AM-colonized plants when Zn was in high supply, which correlates with

Zn isotope data in tomato showing that the mycorrhizal pathway of Zn uptake is not suppressed at high soil Zn concentrations, and is similar regardless of available Zn in the soil (S. Watts-Williams, Smith et al., 2015). This is in contrast to the expression of the AM-specific Pi transporter *MtPT4*, which is strongly down-regulated when P is highly available to the plant (Ferrol et al., 2019), and the transport of isotope labelled P via the mycorrhizal pathway of uptake is likewise suppressed (S.J. Watts-Williams, Jakobsen et al., 2015). Uptake of heavy metals such as Zn, Mn and Fe via the AM pathway might be beneficial to the plant due to the hyphae, rather than the roots, acquiring them, which could reduce the risk of importing unwanted cations such as cadmium (Cd). This may also explain the general down-regulation of non-PAM ZIPs in AM-colonized plants. On the other hand, it is important to note that it is likely that MtZIP14 can transport other divalent cations, such as Fe and Mn. The study of loss of *MtZIP14* function in Fe- and Mn-limited soils will be interesting avenues of future research that will provide a more comprehensive understanding of the role of MtZIP14.

We have identified and described a ZIP transporter that we hypothesize is linked to the function of AM fungal symbiosis in *M. truncatula*, and related to plant Zn nutrition. This information contributes to the development of a comprehensive plant-AM fungal nutrient exchange model, and will stimulate research to identify other transporters involved in AM nutrient acquisition. Ultimately, the expansion of the AM transporter 'toolkit' will allow researchers to identify plant genotypes with highly functional AM fungal nutrient uptake which could, in turn, be used to improve crop nutrition.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. Raw RNA-seq data are available at the NCBI Sequence Read Archive under project ID PRJNA660297. All other data generated or analysed during this study are included in this published article (and its supplementary information files) or available on request to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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