Transcriptional regulation of metal metabolism- and nutrient absorption-related genes in *Eucalyptus grandis* by arbuscular mycorrhizal fungi at different zinc concentrations

Xinyang Wang, Jingwei Liang, Ziyi Liu, Yuxuan Kuang, Lina Han, Hui Chen, Xianan Xie, Wentao Hu* and Ming Tang*

**Abstract**

**Background:** *Eucalyptus* spp. are candidates for phytoremediation in heavy metal (HM)-polluted soils as they can adapt to harsh environments, grow rapidly, and have good economic value. Arbuscular mycorrhizal fungi (AMF) are the most widely distributed plant symbiotic fungi in nature, and they play an important role in promoting the phytoremediation of HM-polluted soils. However, few studies have evaluated the HM detoxification mechanism of *E.* spp. in symbiosis with AMF, and thus, the molecular mechanism remains unclear.

**Results:** The gene transcription and metabolic pathways of *E. grandis* were studied with and without inoculation with AMF and at different zinc (Zn) concentrations. Here, we focused on the transcript level of six HM-related gene families (ZNT, COPT/Ctr, YSL, ZIFL and CE). Under high-Zn conditions, thirteen genes (ZNT:2, COPT/Ctr:5, YSL:3, ZIFL:1, CE:2) were upregulated, whereas ten genes (ZNT:3, COPT/Ctr:2, YSL:3, ZIFL:1, CE:1) were downregulated. With AMF symbiosis under high-Zn conditions, ten genes (ZNT:4, COPT/Ctr:2, YSL:3, CE:1) were upregulated, whereas nineteen genes (ZNT:9, COPT/Ctr:2, YSL:3, ZIFL:4, CE:1) were downregulated. Under high-Zn conditions, genes of three potassium-related transporters, six phosphate transporters (PHTs), and two nitrate transporters (NRTs) were upregulated, whereas genes of four potassium-related transporters, four PHTs, and four nitrogen-related transporters were downregulated. With AMF symbiosis under high-Zn conditions, genes of two potassium-related transporters, six ammonium transporters (AMTs) and five PHTs were upregulated, whereas genes of six potassium-related transporters, two AMTs and five PHTs were downregulated.

**Conclusions:** Our results indicates that AMF increases the resistance of *E. grandis* to high-Zn stress by improving nutrients uptake and regulating Zn uptake at the gene transcription level. Meanwhile, our findings provide a genome-level resource for the functional assignments of key genes regulated by Zn treatment and AM symbiosis in...
Background

Large quantities of heavy metals (HMs), such as zinc (Zn), copper (Cu), iron (Fe), and cadmium (Cd), are often released into the soil as a result of soil acidity, flooding, mining, industrial activities, or other sources of pollution [1]. HM contamination reduces the area of usable land and restricts the distribution of vegetation. Moreover, in tailings areas, the bioaccumulation of heavy metals in plants and animals causes serious harm to the food chain and, consequently, human health. To eliminate HMs from the ecosystem, bioremediation and phytoremediation are the most significant and appropriate strategies [2]. Phytextraction and phytostabilization are the main methods used for phytoremediation [3]. Many plants have been developed for phytoremediation and phytostabilization. For example, Brown et al. [4] discovered that Thlaspi caerulescens super-accumulates Zn and Cd, indicating that the plant has potential remediation capacity in HM-contaminated soil. Blaylock et al. [5] found that mustard greens (Brassica juncea) can absorb and accumulate various HMs, such as lead (Pb), Cd, and Zn, and that Thlaspi can effectively absorb Zn, Pb, and Cd. However, many of the plants currently used for the bioremediation of HM pollution have slow growth and low biomass content [6, 7]. Therefore, to improve remediation efficiency, a feasible direction for the bioremediation of HM-contaminated soil is to screen for plants that are fast-growing, have high biomass content, and are HM tolerant.

Eucalyptus grandis has the characteristics of fast growth, strong stress resistance, and high economic value [8]. Both E. grandis 5 and E. grandis × E. urophylla can adapt to severe environments in mining areas and also have strong phytoremediation potential [9]. In some mining areas polluted with Zn, Eucalyptus spp. have been shown to be HM pollution hyperaccumulators [10]. In the Huangpu District of Guangzhou, where Zn pollution is a serious problem, E. urophylla became the dominant species [11]. These data suggest that Eucalyptus spp. are potential tools to remedy Zn pollution in the soil [12]. Moreover, Eucalyptus spp. can establish symbioses with arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal (ECM) fungi [13]. Campos et al. [14] found AMF/ECM fungi symbioses at all ages of E. grandis and E. urophylla.

Zn can be toxic if it accumulates in excess amounts, although it is also an essential nutrient for plant growth at low concentrations [15]. Zn toxicity often occurs in soils contaminated by mining and smelting activities, in agricultural soils treated with sewage sludge, and in urban soils enriched with anthropogenic Zn inputs [16]. To respond to Zn toxicity, plants require a tightly controlled metal homeostasis network that balances the uptake, utilization, and tolerance of Zn [17, 18]. Zn-related transporters are involved in the absorption, intracellular transport, localization, and efflux of Zn. These include the P1B-type heavy metal ATPase (HMA), Zn- and Fe-regulated transporter-like protein (ZIP), copper transporter (COPT/Ctr), yellow stripe-like (YSL) transporter, Zn-induced facilitator1 (ZIF1) transporter, and cation efflux (CE) transporter families.

There is convincing evidence that mycorrhizal associations are of major importance in reducing metal transfer to plants [19] or serving as an effective exclusion barrier against the transport of these elements from roots to shoots [20]. In addition, mycorrhizal fungi, especially AMF, favor the absorption of nitrogen (N), phosphorus (P), and potassium (K) [21]. AMF also improve the tolerance of plants to HM stress [22], prevent plant contact with HMs, and favor the extraction of HMs from the soil [23]. As many plants form AMF symbioses, even in highly HM-contaminated soils [24], AMF may be an important tool for the remediation of HM-contaminated soils [25]. Inoculation with AMF can improve the nutritional status of Eucalyptus spp. by increasing N, P, and K concentrations and improve the tolerance of Eucalyptus spp. to HM stress [26]. Previous studies have shown that under conditions of Pb stress, AMF symbiosis can increase the uptake of Pb by the shoots of E. globulus [27]. However, few studies have focused on the mechanism of Zn tolerance and enrichment in Eucalyptus spp. with AMF symbiosis. It is necessary to explore the detoxification mechanism of Eucalyptus spp. in response to Zn stress, as this may provide the theoretical basis for the ecological restoration of artificial E. grandis forests in Zn-contaminated areas.

In this study, to better understand the mechanisms of the response to Zn stress in E. grandis with AMF symbiosis, candidate genes were screened using RNA sequencing (RNA-seq) analysis of E. grandis under different zinc chloride (ZnCl₂) concentrations. Important response pathways, genes involved in Zn tolerance, and gene families associated with nutrient absorption were
identified. This aim of this study was to determine (i) the type of metal transporters that are regulated by different Zn concentrations and (ii) the type of HM transporters and nutrient-associated transporters that respond to Zn treatment with AMF symbiosis. These results may contribute to a greater understanding of Zn-responsive mechanisms in *E. grandis* with AMF symbiosis.

**Results and discussion**

In this study, we investigated genes involved in Zn and nutrient absorption by the transcriptome analysis of *E. grandis* with AMF symbiosis under different Zn concentrations, followed by the putative subcellular localization and functional prediction of DEGs. Correlations among genes belonging to the same gene family were also analyzed. After inoculation with AMF under high-Zn conditions, 2253 DEGs were identified, of which 1683 were upregulated and 570 were downregulated. However, only 539 DEGs were identified under high-Zn conditions without AMF inoculation, of which 275 were upregulated and 264 were downregulated (Supplementary Fig. S1). These findings represent the first step in determining the molecular mechanisms of the response of *E. grandis* to high-Zn stress and provide plenty of new candidate genes for studies on tolerant germplasm development and molecular biology in woody plants.

**Translocation and accumulation of Zn in mycorrhizal Eucalyptus**

There were no obvious symptoms of visual toxicity, regardless of metal addition or AMF inoculation. Because the metal treatment was performed after the inoculation, the colonization rate did not change significantly, but the arbuscule abundance decreased significantly and the number of hyphae increased under high-Zn conditions (Supplementary Fig. S2). Compared with the normal Zn concentration, the high Zn concentration caused a 35% reduction in the Pi content of the shoots of *E. grandis*, but the Pi content of the roots did not change significantly (Supplementary Fig. S3). After inoculation with AMF, the Pi content of the shoots increased, indicating that AMF increased the Pi content of *E. grandis* (Supplementary Fig. S3). The pre-experiment shows that *E. grandis* can promote plant growth after inoculation with AMF (Supplementary Fig. S4). These results also indicate that there is an antagonistic relationship between Zn and P in *Eucalyptus* spp. and that mycorrhizal plants may reduce HM-induced damage to plants through P absorption. The interconnection between these two nutrients has been observed in many crop species and can be summarized as follows: Zn-deficient plants over-accumulate Pi in the shoots and, conversely, Pi-deficient plants overaccumulate Zn in the shoots [28]. Mycorrhizae play an important role in the acquisition of P by the host plant [29], and they may also facilitate Zn transport in the soil-fungi-plant continuum [19]. Nevertheless, the molecular basis of the Pi-Zn interaction in plants remains poorly understood in both mycorrhizal and non-mycorrhizal plants [30].

**Transporters that introduce metals into the cytosol**

As seedlings from the NM- and AMF-treatment groups demonstrated distinct patterns of Zn and P contents in their acclimation to Zn availability (Supplementary Fig. S3), specific differences may also be expected in the transcriptional regulation pattern of key genes implicated in Zn metabolism [31]. Therefore, the transcript levels of representative genes involved in Zn acquisition and assimilation were assessed in the roots of plants in the NM- and AMF-treatment groups.

**ZNT family**

ZNTs are micronutrient transporters from the zinc-resistance transporter and iron-resistance transporter-like protein (ZIP) family. ZIP transporters contain a conserved cytosolic histidine-rich loop between transmembrane (TM) domains 3 and 4 in eukaryotes [32], which is associated with metal specificity and metal transport rate [33].

Nineteen candidate ZNT family genes were identified in the *E. grandis* genome. The characteristics of these genes, including the open reading frame length, chromosome location, number of exons, and the molecular weight and isoelectric point of the encoded protein were analyzed and are shown in Supplementary Table 1. To identify the evolutionary relationships between *EgZNT* genes of different plant species, a phylogenetic tree was constructed with 86 ZIP transport protein sequences from 10 plant species (Supplementary Fig. S5). Bioinformatics analyses showed that ZNT genes were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S6). And the chromosomal location of ZNT gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S7).

Cluster analysis of transcript levels of the ZNT gene family clearly separated root samples from NM- and AMF-treatment groups based on their responsiveness to Zn (Fig. 1). Nine ZNT genes (*ZNT-Eucgr.A00916, ZNT-Eucgr.E01913, ZNT-Eucgr.A00921, ZNT-Eucgr.K01345, ZNT-Eucgr.K01348, ZNT-Eucgr.E01901, ZNT-Eucgr.K01343, ZNT-Eucgr.F02059,* and *ZNT-Eucgr.F02060*) formed subcluster I. Under high-Zn conditions, the abundance of the transcripts of subcluster I genes in roots from the AMF-treatment group was higher than or similar to that in roots from the NM-treatment group.
The second subcluster of genes included ZNT-Eucgr.K01344, ZNT-Eucgr.F02058, ZNT-Eucgr.D01644, ZNT-Eucgr.A00918, ZNT-Eucgr.C00648, ZNT-Eucgr.D01642, ZNT-Eucgr.E01090, ZNT-Eucgr.K01349, and ZNT-Eucgr.E01082. The transcript levels of these genes were lower in roots from the AMF-treatment group than in roots from the NM-treatment group under low-Zn conditions.

Limiting the Zn supply affected the transcript levels of genes involved in Zn metabolism in the roots from both the AMF- and NM-treatment groups. Generally, the transcript levels of genes from subcluster I were higher in roots from the AMF-treatment group than in those from the NM-treatment group under low-Zn conditions (Fig. 1). However, the transcriptional pattern of ZNT-Eucgr.E01913 was completely different between the NM- and AMF-treatment groups. The transcript levels of this gene were upregulated in roots from the NM-treatment group under low- and high-Zn conditions, whereas in roots from the AMF-treatment group, they were only upregulated under high-Zn conditions. We found that ZNT-Eucgr.E01913, OsIRT1, HvIRT1, AtIRT1, MtZIP1, and MtZIP6 were in the same branch of the phylogenetic tree (Supplementary Fig. S5). HvIRT1 is involved in Zn absorption under conditions of Zn deficiency [34]. IRT1 is considered to be the main transporter for Fe²⁺ uptake in roots and is polarly localized to the soil-facing side of the epidermal plasma membrane [35]. AtIRT1 is involved in Fe uptake. Barberon et al. [35] showed that plants growing under high Zn concentrations are deficient in iron. Recently, a ZIP transporter gene (MtZIP6) in the model legume Medicago truncatula was identified as being significantly upregulated by AMF colonization when Zn was at a limiting concentration [36]. The ZIP transporter encoded by MtZIP6 is located in the plasma membrane and is involved in Zn transport in rhizobial symbioses [37]. However, MtZIP6 has been shown to transport Fe in addition to Zn [38]. In contrast, the lower affinity of MtZIP1 for Zn suggests that this transporter plays a role in Zn transport within the plant, because the concentration of Zn has been shown to be much higher in plant compartments than in the soil [39]. Therefore, the increase in the expression levels of ZNT-Eucgr.E01913 under low-Zn conditions suggested that it is also involved in iron absorption under conditions...
of Zn deficiency. The increase in the expression levels of ZNT-Eucgr.E01913 under high-Zn conditions may be caused by iron deficiency. This result also suggests that AMF may assist plants to absorb Zn through other mechanisms at low Zn concentrations. Thus, ZNT-Eucgr.E01913 may be involved in regulating the zinc-iron balance in plant roots.

The expression of ZNT-Eucgr.K01343, which is homologous to AtZIP1 (Supplementary Fig. S5), from the ZIP family, was induced under conditions of Zn deficiency and was inhibited under high-Zn conditions (Fig. 1). AtZIP1 serves as a vacuolar transporter that remobilizes Zn from the vacuole to the cytoplasm in root cells [40]. AtZIP1 expression is mainly induced under conditions of Zn deficiency and is involved in Zn uptake and redistribution [41]. Thus, ZNT-Eucgr.K01343 may also be involved in Zn uptake and redistribution under conditions of Zn deficiency.

ZNT-Eucgr.F02059 and ZNT-Eucgr.F02060 have a high degree of homology and are in the unified branch of the phylogenetic tree with OsZIP2, MtZIP7, and AtZIP2 (Supplementary Fig. S5). Milner et al. [40] reported that Zn uptake from the root apoplast is carried out by AtZIP2 in the root endodermis of Arabidopsis thaliana. AtZIP2 is a Zn uptake transporter that is located mainly in the root stele. The expression levels of ZNT-Eucgr.F02059 and ZNT-Eucgr.F02060 were low under NM conditions and were not affected by the Zn concentration. The levels of these two transcripts increased significantly after AMF inoculation, but their levels decreased with the increase in Zn concentration in plants with AM treatment. MtZIP7, a putative Zn and manganese (Mn) transporter, is expressed in both arbuscule-colonized and adjacent non-colonized cortical cells [42].

Our results showed that the expression of ZNT-Eucgr.F02059 and ZNT-Eucgr.F02060 is specifically induced by mycorrhizae. AMF contribute to Zn uptake by their host plants and alleviate HM toxicity. Therefore, ZNT-Eucgr.F02059 and ZNT-Eucgr.F02060 were specifically induced by AMF symbiosis, and their encoded proteins may transport Zn released by the AMF at the symbiotic interface.

Of the ZNTs in subcluster II, the transcript abundance of ZNT-Eucgr.D01642, ZNT-Eucgr.D01644, ZNT-Eucgr.E01082, and ZNT-Eucgr.E01090 decreased after inoculation with AMF under high-Zn conditions (Fig. 1). These genes are in the same branch of the phylogenetic tree and are highly homologous, but only ZNT-Eucgr.D01644 was induced by a high Zn concentration without AMF. These four transporters are in the same subfamily as MtZIP2 and AsZIP2 in the phylogenetic tree (Supplementary Fig. S5). The expression level of AsZIP2 has been shown to increase with increasing Zn concentration [43]. ZNT-Eucgr.D01644 may have the same function as MtZIP2 to transport excess Zn from the intercellular space into the vacuole and thus maintain a suitable cytoplasmic Zn concentration [44]. As shown in Fig. 1, AMF symbiosis effectively inhibited the transcript levels of this subfamily, presumably because AMF symbiosis inhibits excessive Zn absorption [44]. There are two possible explanations for this phenomenon. First, AM fungal hyphae bind and fix HMs on the fungal cell wall, thus reducing the absorption of heavy metals by the host plant. Second, AMF deposit and chelate HMs in the rhizosphere [45].

We performed Pearson's correlation analysis of the ZNT family genes and found that the transcript levels of ZNT-Eucgr.K01343 and ZNT-Eucgr.F02060 ($R=0.739$), ZNT-Eucgr.F02059 and ZNT-Eucgr.F02060 ($R=0.953$), ZNT-Eucgr.D01644 and ZNT-Eucgr.F02058 ($R=0.765$), and ZNT-Eucgr.K01344 and ZNT-Eucgr.E01901 ($R=0.78$) were highly significantly correlated ($P<0.001$, Supplementary Table. S2).

COPT/Ctr transporters

Cu transporters are known as COPTs in plants [46] and Ctrs in animals and fungi [47]. In plants, COPTs have been suggested to play a role in Cu uptake from the soil and Cu delivery to pollen [48]. The PtCOPT gene of Populus trichocarpa is expressed in many tissues and may be involved in regulating plant development [49]. Previous studies have indicated that the COPT/Ctr gene family in animals and herbaceous plants is induced during deficiencies and excesses of Cu [50] and by Fe, Mn, and Zn stress [51].

Twenty-one candidate COPT/Ctr family genes were identified in the E. grandis genome. Heat maps showed that COPTs were divided into two subclusters (Fig. 2). In roots, COPT-Eucgr.E04213, COPT-Eucgr.A00965, COPT-Eucgr.B03028, COPT-Eucgr.E00718, COPT-Eucgr.E00673, COPT-Eucgr.L01980, Ctr-Eucgr.B02736, COPT-Eucgr.A02786, COPT-Eucgr.D01867, COPT-Eucgr.H01538, COPT-Eucgr.L03723, COPT-Eucgr.A00960, and COPT-Eucgr.A00963 formed subcluster I (Fig. 2). Subcluster I was divided into two small clusters. The transcript abundance of COPT-Eucgr.E04213, COPT-Eucgr.A00965, COPT-Eucgr.B03028, COPT-Eucgr.E00718, COPT-Eucgr.E00673, and COPT-Eucgr.L01980 in subcluster I was higher under low- and high-Zn conditions in both the NM- and AMF-treatment groups compared with their levels under normal-Zn condition. The expression levels of genes in the other cluster were diverse. For example, the expression level of Ctr-Eucgr.B02736 induced by AMF increased significantly under both low- and high-Zn conditions, and its expression level under high-Zn conditions decreased after NM and AMF treatments.
The second subcluster consisted of COPT-Eucgr.E00765, COPT-Eucgr.03666, Ctr-Eucgr.J01987, COPT-Eucgr.A02727, COPT-Eucgr.E04208, Ctr-Eucgr.J01988, COPT-Eucgr.B02737, and Ctr-Eucgr.J01992. The transcript levels of these genes, except COPT-Eucgr.E00765 and COPT-Eucgr.03666, were similar or lower after NM and AMF treatments under low-Zn conditions compared with their levels under normal-Zn conditions (Fig. 2).

Most members of the COPT gene family of *E. grandis* were in the same branch of the phylogenetic tree, indicating that the genes of this family are highly conserved in *E. grandis* (Supplementary Fig. S8).

Based on a comparison of transcriptome and JGI data, two groups of Ctrs and COPTs were identified in *E. grandis*. Sixteen of these genes were classified as COPTs and five were classified as Ctrs (Fig. 2). The five Ctr transporters had a high degree of homology with the previously reported PtCOPT gene of *P. trichocarpa*. In all COPT/Ctr transporters, motif 1 and motif 3 corresponded to the TM sites. It is noteworthy that motif 1 contained both the MxxxM and GxxxG motifs (Supplementary Fig. S9). The MxxxM motif is essential for Cu acquisition [52], and the conserved GxxxG motif is essential for trimerization in hCtr1 [53]. Previous studies of COPT/Ctr families in animals and herbaceous plants have demonstrated their upregulation during deficiencies and excesses of Cu [50] and in response to Fe, Mn, and Zn stress [51]. And the chromosomal location of COPT/Ctr gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S10).

Heatmap analysis revealed that the expression of EgCOPT genes was induced under both limited and excessive Zn concentrations (Fig. 2). This result is consistent with the PtCOPT expression pattern previously identified in *P. tomentosa* [49]. Quantitative reverse transcription-PCR analysis has shown that the expression of PtCOPT genes is induced under conditions of limited and excessive Zn [49]. After inoculation with AMF, under low-Zn conditions, EgCOPT promoted the absorption of Zn and increased the Zn concentration in the plant, resulting in decreased expression levels of EgCOPTs. Under high-Zn conditions, mycorrhizal fungi can absorb excess Zn, reduce the Zn concentration in the plant, and reduce the expression level of EgCOPT (Fig. 2). This result indicates that mycorrhizal fungi can maintain the balance between HM ions in the host plant, thereby reducing the impact of HM ions. Under Zn stress, mycorrhizal fungi
have been shown to downregulate plant Zn transporters to promote homeostasis [54].

We performed Pearson’s correlation analysis of the COPT/Ctr family genes and found that the transcript levels of COPT-Eucgr.A00965 and COPT-Eucgr.E00673 ($R = 0.772$) and COPT-Eucgr.E00765 and COPT-Eucgr.E04213 ($R = 0.748$) were highly significantly correlated ($P < 0.001$, Supplementary Table. S3).

**YSL transporter family**

YSL family transporters belong to the oligopeptide transporter family and are significant iron transport proteins. YSL transporters do not use free metals as substrates, but complexes of metals with nicotiana amine (NA) or its derivatives [55]. NA is a non-proteogenic amino acid that is synthesized from S-adenosyl-methionine by the enzyme NA synthase [56](NAS). Transport by YSL proteins is induced by H$^+$-symport [57]. Additionally, at least some plant oligonucleotide transporters are also associated with metal transport [58], although the identity of the metal complex transported remains elusive. Little is known about the structure of these proteins, with different models proposing a range of 11–16 TM regions [58]. In broad terms, YSL transporters are involved in metal uptake from the soil in monocots and in long-distance metal distribution in both monocots and dicots [59].

Nineteen candidate YSL family genes were identified in the *E. grandis* genome. Limiting the supply of Zn affected the transcript levels of genes involved in Zn metabolism in the roots of both AMF- and NM-treatment groups. In roots, YSL-Eucgr.I01628, YSL-Eucgr.A01430, and YSL-Eucgr.K00010 formed subcluster I (Fig. 3). Under high-Zn conditions, the transcript abundance of genes in subcluster I was higher after NM treatment than after AMF treatment (Fig. 3). The second subcluster consisted of YSL-Eucgr.K02316, YSL-Eucgr.K02315, YSL-Eucgr.K02319, YSL-Eucgr.K00012, YSL-Eucgr.D01684, YSL-Eucgr.H00652, YSL-Eucgr.H00651, YSL-Eucgr.K00413, YSL-Eucgr.B00833, YSL-Eucgr.K02320, YSL-Eucgr.K02321, YSL-Eucgr.B00295, YSL-Eucgr.B00296, YSL-Eucgr.B00835, YSL-Eucgr.G02568, and YSL-Eucgr.K02318. Under high-Zn conditions, the transcript levels of these genes were similar

![Fig. 3 Heatmap of YSL family members. Heatmap of YSL family members. Cluster analysis of transcriptional fold-changes of COPT/Ctr family gens in roots of non-mycorrhizal and mycorrhizal *E. grandis* exposed to 0.01, 0.5, or 150 μM ZnCl$_2$. The color scale indicates fold-changes of mRNAs. For each gene, the expression levels in non-mycorrhizal roots exposed to 0.5 μM ZnCl$_2$ were defined as 1, and the corresponding fold-changes under 0.01 and 150 μM ZnCl$_2$ were calculated.](image-url)
or lower after NM treatment compared with their levels after AMF treatment.

To identify the evolutionary relationships between *Eg*YSL genes of different plant species, a phylogenetic tree was constructed with 50 YSL transport protein sequences from 5 plant species (Supplementary Fig. S11). Bioinformatics analyses showed that YSL genes were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S12). And the chromosomal location of YSL gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S13).

We found that the expression level of *YSL-Eucgr.*K00010 was significantly increased under high-Zn conditions without AMF inoculation but significantly decreased under high-Zn conditions after with AMF inoculation (Fig. 3). Comparatively, *YSL-Eucgr.*K00010 expression levels increased after inoculation with AMF under low-Zn conditions. This result showed that after inoculation with AMF, the expression of this gene can be promoted when Zn concentrations are low and inhibited when Zn concentrations are high. Therefore, this gene may play a role in both nutrient absorption and metal resistance.

The expression level of *YSL-Eucgr.*K02319 increased significantly after inoculation with AMF under normal- and high-Zn conditions, and its expression levels decreased after both NM and AMF treatments under high-Zn conditions. We found that *YSL-Eucgr.*K02319 was in the same branch of the phylogenetic tree as *Tc*YSL5 and *Tc*YSL7 (Supplementary Fig. S11). In situ hybridization showed that *Tc*YSL7 and *Tc*YSL5 are expressed around the vasculature of the shoots and in the central cylinder in the root [60]. Exposure to HMs (Zn, Cd, and nickel (Ni)) does not affect the high levels of constitutive expression of *Tc*YSL genes [60] (Gendre et al., 2007). We found that *YSL-Eucgr.*K02319 was induced by AMF and was affected by changes in the Zn concentration, indicating that this gene may be involved in the transport of HM and homeostatic balance.

We performed Pearson’s correlation analysis of genes in the YSL family and found that the transcript levels of *YSL-Eucgr.*K02319 and *YSL-Eucgr.*K02321 were highly significantly correlated ($R = 0.924$, $P < 0.001$; Supplementary Table. S4).

**ZIFL transporter family**

ZIFL genes are major facilitator superfamily (MFS) transporters that play roles in responding to different stresses, including Zn stress [61]. Since the identification of three ZIFL genes in *Arabidopsis*, referred to as *AtZIF1* (AT5G13740), *AtZIFL1* (AT5G13750), and *AtZIFL2* (AT3G43790), evidence for their role in Zn homeostasis has been accumulating [61]. ZIF1 is thought to be involved in the proton-coupled transport of metal chelators or metal-chelate complexes into vacuoles, as the ZIF1 protein contains conserved motifs for proton/substrate antiport and related proteins mostly transport organic molecules [62].

Eight candidate ZIFL family genes were identified in the *E. grandis* genome. To identify the evolutionary relationships between *Eg*ZIFL genes from different plant species, a phylogenetic tree was constructed with 35 ZIFL transport protein sequences from five plant species (Supplementary Fig. S14). Bioinformatics analyses showed that ZIFL genes were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S15). And the chromosomal location of ZIFL gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S16).

ZIFL genes were found to have different evolutionary histories in monocot and dicot lineages, which is consistent with the conclusions of Ricachenevsky et al. [63].

We analyzed the expression of *Eg*ZIFL genes with different Zn treatments, between mycorrhizal and non-mycorrhizal plants, and in response to different Zn concentration stresses. The data on the expression of *Eg*ZIFL genes under different Zn concentrations for NM- and AMF-treated plants are shown in Fig. 4.

*Eg*ZIFL1, *AtZIF1*, *AtZIFL1*, and *PtZIFL2* were in a unified position on the evolutionary tree (Supplementary Fig. S14). The essential micronutrients Fe and Zn often limit plant growth but are toxic in excess. *PtZIFL2* was predicted to have a typical conserved domain (MFS structure and zinc finger structure), indicating that it is a hydrophobic TM secreted protein with a TM transport function when Zn is in excess. Through subcellular localization prediction analysis, these four proteins in *P. trichocarpa* were localized to the plasma membrane, while *AtZIF1* was localized to the vacuole membrane [63, 64]. The *AtZIF1* transporter is clearly involved in Zn homeostasis, as the loss-of-function *atzif1* mutant has altered Zn distribution and its transcription is upregulated by excess Zn [65]. We found that *ZIFL-Eucgr.*G02630 was upregulated by a high Zn concentration (Fig. 4), which was consistent with the results of previous studies of this gene in *Arabidopsis*. This result indicates that *ZIFL-Eucgr.*G02630 may be involved in Zn homeostasis and may thus be related to HM resistance in *Eucalyptus* spp. After inoculation with AMF at a high Zn concentration, the expression level of *ZIFL-Eucgr.*G02630 decreased significantly, indicating that AMF is involved in maintaining a steady state of Zn to facilitate the resistance of *Eucalyptus* spp. to HMs.
We performed Pearson’s correlation analysis of the ZIFL family genes and found that the transcript levels of ZIFL-Eucgr.G02629 and ZIFL-Eucgr.L01874 ($R = 0.829$) were highly significantly correlated ($P < 0.001$, Supplementary Table. S5).

Transporters that remove metals from the cytosol

HMA transport family

HMAs are proteins that hydrolyze ATP and use the released energy for TM transport. They also transport $\text{Zn}^{2+}$, $\text{Cd}^{2+}$, $\text{Pb}^{2+}$, $\text{Cu}^{2+}$, and other heavy metal ions across the membrane. HMA proteins transport heavy metal ions selectively. They may play an important role in the phytoremediation of contaminated soil. HMA family members are grouped into two distinct clades in phylogenetic analyses [66]. Members of one clade play roles in Cu and silver (Ag) transport, while members of the second clade function as Zn/cobalt (Co)/Cd/Pb transporters [67].

In roots, the abundance of HMA family gene transcripts was higher after NM treatment than after AMF treatment under low-Zn conditions, except for HMA-Eucgr.J00786 transcript levels, which were higher after AMF treatment. Every member of the HMA gene family had lower transcript levels after AMF treatment than after NM treatment under high-Zn conditions (Fig. 5).

To identify the evolutionary relationships between HMA genes of different plant species, a phylogenetic tree was constructed with 32 HMA transport protein sequences from 4 plant species (Supplementary Fig. S17). Bioinformatics analyses showed that HMA genes were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S18). And the chromosomal location of HMA gene family were shown in the chromosome-scale genome of $E.\text{grandis}$ (Supplementary Fig. S19).

HMA-Eucgr.C00131 and HMA-Eucgr.C02245 were found to be in the same branch of the phylogenetic tree as AtHMA2 and OsHMA2 (Supplementary Fig. S17). Therefore, these two genes are predicted to have the same function as members of the HMA2 subfamily.

OsHMA2 plays a role in Zn and Cd loading to the xylem and participates in root-to-shoot translocation of these metals in rice [68]. Eren et al. [69] found that AtHMA2 is a high-affinity Zn transporter, transporting Zn out of cells to maintain a low Zn level in the cytoplasm and a

Fig. 4 Heatmap of ZIFL family members. Cluster analysis of transcriptional fold-changes of ZIFL family gens in roots of non-mycorrhizal and mycorrhizal $E.\text{grandis}$ exposed to 0.01, 0.5, or 150 $\mu\text{M}$ ZnCl$_2$. The color scale indicates fold-changes of mRNAs. For each gene, the expression levels in non-mycorrhizal roots exposed to 0.5 $\mu\text{M}$ ZnCl$_2$ were defined as 1, and the corresponding fold-changes under 0.01 and 150 $\mu\text{M}$ ZnCl$_2$ were calculated.
steady-state balance of Zn within cells. The pericycle plasma membrane protein AtHMA2 is responsible for loading Zn into the xylem, thus contributing to the control of the long-distance transport of Zn from roots to shoots [70]. The decrease in HMA-Eucgr.C00131 and HMA-Eucgr.C02245 gene expression levels under high-Zn conditions decreased the accumulation of Zn in the roots of E. grandis. After inoculation with AMF under high-Zn conditions, the expression of these genes was significantly inhibited, which may be because AMF symbiosis directs HMs to the roots and HM transporters at the plasmalemma or tonoplast of both symbiotic partners may catalyze the export of HMs from the cytoplasm [71]. AtHMA1 is located in the chloroplast envelope, where it contributes to Zn detoxification by reducing the Zn content in A. thaliana plastids under conditions of excess Zn [73]. Therefore, HMA-Eucgr.J00227 is considered to have a Zn transport function, and its expression level decreased under high-Zn conditions. This may have been a result of Zn redistribution, indicating that this gene may have a detoxification function. After inoculation with AMF, HMs may bind to the cell wall and may be deposited in the vacuole of the fungus, which reduces the absorption of Zn by the plant. Thus, the expression level of this gene decreased after inoculation with AMF.

We found that HMA-Eucgr.J00786 was induced by a high Zn concentration (Fig. 5), which was consistent with the expression pattern of OsHMA9 (Supplementary Fig. S17). OsHMA9 localizes to the plasma membrane and can discharge heavy metals to the outside of the cell, which may play a role in HM detoxification [74]. After inoculation with AMF, the expression level of HMA-Eucgr.J00786 increased when the Zn concentration was low. The resulting promotion of Zn efflux from root cells then allows more Zn to be transported to the soil. The decrease in expression under high-Zn conditions may be due to the AM fungal absorption of Zn transported from plant cells.

We performed Pearson’s correlation analysis of HMA family genes and found that the transcript levels of

Fig. 5 Heatmap of HMA family members. Cluster analysis of transcriptional fold-changes of HMA family gens in roots of non-mycorrhizal and mycorrhizal E. grandis exposed to 0.01, 0.5, or 150 μM ZnCl₂. The color scale indicates fold-changes of mRNAs. For each gene, the expression levels in non-mycorrhizal roots exposed to 0.5 μM ZnCl₂ were defined as 1, and the corresponding fold-changes under 0.01 and 150 μM ZnCl₂ were calculated.
HMA-Eucgr.C00131 and HMA-Eucgr.D01863 were highly significantly correlated ($R = 0.773$, $P < 0.001$, Supplementary Table. S6).

**The cation efflux gene family**

The CE gene family, also known as the cation diffusion facilitator gene family, was first described by Nies and Silver [75] and is considered to be compatible with Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ transport. Members of this family are associated with the HM tolerance of plants. Most CE transporters are involved in storing metal ions within cells and transporting metal ions out of cells [76]. Many CE gene family members have been identified, and they have certain common structural features, including a C-terminal cation-binding domain, an N-terminal signal peptide sequence, and approximately six TM domains. The TM domains TM4-TM5 in CE family members in eukaryotes are all rich in histidine [77]. Most of the CE proteins are located in the cell membrane, but some are located in intracellular membrane systems, such as vacuole membranes and Golgi membranes.

To identify the evolutionary relationships between CE genes of different plant species, a phylogenetic tree was constructed with 44 CE transport protein sequences from 6 plant species (Supplementary Fig. S20). Bioinformatics analyses showed that CE genes were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S21). And the chromosomal location of CE/MTP gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S22).

Fourteen CE transporters were identified in the *E. grandis* genome based on sequencing results. Cluster analysis of transcript levels of the CE gene family clearly separated root samples from NM- and AMF-treated plants based on their responsiveness to Zn concentrations. According to variations in their transcript levels, they were divided into two subclusters. The two sub-clusters were distinguished by changes induced by AMF inoculation at a high Zn concentration (Fig. 6).

We found that CE-Eucgr.E01084, CE-Eucgr.E01089, and CE-Eucgr.E01088 were highly homologous and were in the same branch of the phylogenetic tree as OsMTP1,
OsMTP11 is a trans-Golgi network-localized Mn transporter that transfers excess Mn from the cytoplasm to the vacuole and reduces the transport of Mn from the roots to the shoots [80]. We found that CE-Eucgr.E01088 and CE-Eucgr.E01084 transcript levels were upregulated after NM treatment under high-Zn conditions (Fig. 6), indicating that these two genes may have a similar function to AtMTP3 in Arabidopsis (Supplementary Fig. S20), which transports excess Mn to the vacuoles in the roots. We conclude that CE-Eucgr.E01088 and CE-Eucgr.E01084 play crucial roles in avoiding Zn stress in the root meristematic tissue before the activation of Zn export systems and the synthesis of phytochelatins. After inoculation with AMF under high-Zn conditions, the transcriptional levels of CE-Eucgr.E01084, CE-Eucgr.E01089, and CE-Eucgr.E01088 were suppressed. This may be because the mycelium increased Zn absorption under high-Zn conditions, resulting in the adsorption of more Zn to the cell wall of mycorrhizal fungi, thus reducing the absorption of Zn by plants [79].

We found that CE-Eucgr.J01168 was highly homologous to AtMTP11 (Supplementary Fig. S20). Eucgr.J01168 had the highest transcriptional level at a low Zn concentration and the transcript levels of these genes were higher after AMF treatment than after NM treatment (Fig. 6). AtMTP11 is involved in the tolerance of A. thaliana to high Mn concentrations. It is localized to the Golgi apparatus and is thought to remove Mn from the roots to the shoots [78]. OsMTP11 is a trans-Golgi network-localized Mn transporter that is required for Mn homeostasis and contributes to the Mn tolerance of rice [81]. Therefore, we predicted that Eucgr.J01168 may also be located in the Golgi apparatus. It was induced under low-Zn conditions and promoted the transport of Zn to the shoots, whereas under high-Zn conditions, it may be transported out of cells, thereby playing a role in cellular metal homeostasis.

We performed Pearson’s correlation analysis of CE/MTP family genes and found that the transcript levels of CE-Eucgr.F04469 and CE-Eucgr.A02454 ($R = 0.849$), CE-Eucgr.J01747 and CE-Eucgr.F04468 ($R = 0.764$), and CE-Eucgr.K00323 and CE-Eucgr.K01853 ($R = 0.762$) were highly significantly correlated ($P < 0.001$, Supplementary Table. S7).

**Improved metal tolerance through changes in nutrients**

**Phosphorus transporter genes**

Phosphorus is an essential element for plants. It is involved in the synthesis of nucleic acids and ATP in cells, as well as the regulation of enzyme activity and signal transduction [82]. Pi is relatively inaccessible to plant roots because of its low solubility and high capacity for adsorption to soil particles. Plants must, therefore, use a complex series of Pi transporters to acquire Pi from the soil and distribute it to tissues and subcellular organelles. Pi acquisition at the root periphery in plants is coupled with proton entry (Pi:$H^+$ symporter) and mediated by members of the PHT1 gene family [83].

Pi-deficient plants over-accumulate Zn in the shoots and, conversely, Zn-deficient plants overaccumulate Pi in the shoots [28]. More than 90% of land plants form symbiotic associations with mycorrhizal fungi [83]. Mycorrhizae play an important role in the acquisition of P by the plant [29] and also facilitate Zn transport in the soil-fungi-plant continuum [19].

Thirty-five candidate PHT family genes were identified in the *E. grandis* genome. Bioinformatics analyses showed that PHT genes were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S23). And the chromosomal location of PHT gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S24). Through a heatmap analysis, we found that PT-Eucgr.A02668, PT-Eucgr.K00323, and PT-Eucgr.J00101 were only expressed after inoculation with AMF. The expression levels of these genes were significantly reduced by a high Zn concentration. The expression level of PT-Eucgr.A02668 was also reduced by a low Zn concentration. However, the expression levels of PT-Eucgr.F03590, PT-Eucgr.I01609, and PT-Eucgr.H03069 increased significantly after inoculation with AMF under high-Zn conditions. Moreover, PT-Eucgr.F03590 and PT-Eucgr.H03069 expression levels were affected by NM treatment under low-Zn conditions (Supplementary Fig. S25).

PT-Eucgr.A02668, PT-Eucgr.K00323, and PT-Eucgr.J00101 showed homology with MtPT4 and OsPT11 (Supplementary Fig. S26). MtPT4 of *Medicago truncatula* and OsPT11 of *Oryza sativa* are localized in the periarbuscular membrane [84, 85]. Moreover, MtPT4 RNAi lines and loss-of-function mutants fail to show symbiosis-associated increases in Pi or growth responses, indicating that MtPT4 is required for Pi transport in the symbiotic system [86]. These data indicate that these three genes are phosphorus transporters specifically induced by mycorrhizal fungi and that they are affected by the Zn concentration.

PT-Eucgr.F03590, PT-Eucgr.I01609, and PT-Eucgr.H03069 showed homology with LePT2 and MtPT2 (Supplementary Fig. S26). Expression of these genes was induced under high-zinc conditions and increased after inoculation with AMF. The expression levels of PT-Eucgr.F03590 and PT-Eucgr.H03069 increased after NM treatment under low-Zn conditions. This result shows that after inoculation with AMF, the absorption
of phosphorus is increased to reduce the toxic effect of heavy metals. PT-Eucgr.H03067 also showed homology with LePT2 and MtPT2. However, PT-Eucgr.H03067 levels increased under high-Zn conditions after NM treatment and after inoculation with AMF; however, under these conditions, the expression levels were lower than those observed after NM treatment under low-Zn and high-Zn conditions. LePT2 is predominantly expressed in Pi-deficient roots and is significantly downregulated in mycorrhizal roots under low-Pi conditions [87]. Therefore, in this sub-branch, the Eucalyptus PHT gene has evolved two functions to cope with the effects of changes in Zn concentration. The expression levels of MtPT2 within mycorrhizal roots are likely influenced by changes in the Zn status of the plant as a result of symbiotic function, as expression levels in the roots are inversely correlated with the P status of mycorrhizal plants [54].

We performed Pearson’s correlation analysis on PHT family genes and found that the transcript levels of PHT-Eucgr.H03069 and PHT-Eucgr.F03590 (R = 0.894) and PHT-Eucgr.H03069 and PHT-Eucgr.I01609 (R = 0.861) were highly significantly correlated (P < 0.001, Supplementary Table. S8).

**AMTs and NRTs**

Plants absorb and utilize two inorganic nitrogen sources, ammonium nitrogen (NH₄⁺) and nitrate nitrogen (NO₃⁻) [88]. The NRT family is responsible for transporting NO₃⁻ and the AMT family is responsible for transporting NH₄⁺ [89, 90].

Fourteen AMTs and eleven NRTs were identified in the *E. grandis* genome based on the sequencing results. Bioinformatic analyses showed that AMTs and NRTs were well conserved and had similar physicochemical properties. Conserved motif structures were also detected (Supplementary Fig. S27). And the chromosomal location of AMT and NRT gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S28).

The expression levels of NRT-Eucgr.H02533 decreased with increasing Zn concentration under NM treatment conditions, and its expression levels increased significantly after inoculation with AMF under high-Zn conditions. Based on the heat map, AMT-Eucgr.K01403 and AMT-Eucgr.K03320 are AMTs that are specifically induced by mycorrhizal fungi, as their expression levels decreased significantly after inoculation with AMF (Supplementary Fig. S29). Zn deficiency reduces the nitrogen content in the root system and increases the non-protein nitrogen content, which mainly affects RNA metabolism and consequently protein synthesis. The large accumulation of free amino acids, regardless of Zn content, causes a decrease in the levels of ammonium nitrogen and nitrate nitrogen [91]. Thus, N is critical for the uptake and accumulation of Zn in plants, and it deserves special attention in the biofortification of food crops with Zn [92].

We found that the expression levels of AMT family genes were decreased by a high Zn concentration and increased significantly after AMF inoculation. However, AMT gene expression levels induced by a low Zn concentration increased after inoculation with AMF, indicating that AMTs play roles in symbiosis with AMF at low Zn concentrations. Thus, nutrient absorption plays a role in improving the resistance of plants to high Zn concentrations.

We performed Pearson’s correlation analysis of genes in the AMT and NRT families and found that the transcript levels of AMT-Eucgr.B02160 and AMT-Eucgr.L03045, AMT-Eucgr.C01787 and AMT-Eucgr.H05067, and AMT-Eucgr.I02296 and AMT-Eucgr.K03320 were highly significantly correlated (P < 0.001, Supplementary Table. S9).

**Potassium transporters**

Potassium transporters are important for potassium uptake by plants. Based on their protein structure and function, they can be divided into the K⁺ uptake permease (KUP)/high-affinity K⁺ (HAK)/KT family, the HKT family, and the cation/proton antiporter (CPA) family, all of which are expressed in different plant tissues or organs. Cation and pH homoeostasis is regulated by monovalent CPAs that fall into two categories, the CPA1 family, which includes Na⁺/H⁺ (CHX) and K⁺ efflux antiporters (KEAs) [93].

Fifty-six candidate potassium transporter genes were identified in the *E. grandis* genome. Among them, the KUP/HAK/KT family was predicted to have 30 members (KTs: 4, HAKs: 23, CHXs: 23), and the CPA family was predicted to have 22 members (CHXs: 17, KEAs: 4, NHXs: 1). Bioinformatic analyses showed that subfamily of potassium transporters were well conserved and had similar physicochemical properties of each subfamily. Each subfamily conserved motif structures were also detected (Supplementary Fig. S30). And the chromosomal location of potassium transporters gene family were shown in the chromosome-scale genome of *E. grandis* (Supplementary Fig. S31). According to the heat map, 9 genes were induced by Zn and mycorrhizae (KTs: 1, KUPs: 2; HAKs: 4; CHXs: 2) (Supplementary Fig. S32).

A high Zn concentration reduced the levels of HAK-Eucgr.C02191, HAK-Eucgr.C00265, and HAK-Eucgr.G02011 transcripts in NM-treated roots. AMF colonization increased the levels of HAK-Eucgr.C02191 transcripts and reduced the levels of HAK-Eucgr.C00265.
and HAK-Eucgr.G02011 transcripts. However, a high Zn concentration increased the levels of HAK-Eucgr.F02234, NHX-Eucgr.F00635, and HAK-Eucgr.G01991 transcripts. Moreover, AMF colonization increased the levels of HAK-Eucgr.F02234 transcripts and reduced the levels of HAK-Eucgr.G01991 and NHX-Eucgr.F00635 transcripts (Supplementary Fig. S32).

Many previous studies have found that certain physiological processes that are important for plant growth, such as cell stretching and shock motion, are also related to the regulation of potassium ions in plants to maintain cell turgor and osmotic potential [94]. In summary, the effect of potassium ions on the regulation of plant cell turgor and osmotic potential has important physiological significance in the normal growth and development of plants.

We performed Pearson's correlation analysis of potassium transporter genes and found that the transcript levels of KT-Eucgr.C04163 and KUP-Eucgr.B03355 (R = 0.821), KUP-Eucgr.B03355 and HT-Eucgr.C04163 (R = 0.821), KUP-Eucgr.B03355 and HAK-Eucgr.C02265 (R = 0.744), KUP-Eucgr.B03355 and HAK-Eucgr.G02011 (R = 0.889), KUP-Eucgr.B03355 and CHX-Eucgr.K03153 (R = 0.750), HAK-Eucgr.C02265 and HAK-Eucgr.G02011 (R = 0.747), HAK-Eucgr.C02265 and CHXEucgr.E00818 (R = 0.861), HAK-Eucgr.C02265 and CHXEucgr.K03153 (R = 0.939), CHXEucgr.E00818 and HAK-Eucgr.G02011 (R = 0.733), and CHXEucgr.E00818 and CHXEucgr.K03153 (R = 0.771) were highly significantly correlated (P < 0.001, Supplementary Table. S10).

**PCA of heavy metal transporter and nutrient transporter responses**

A PCA was performed using the data of related genes involved in HM and nutrient responses. In the PCA plot, a greater distance between symbols associated with Zn concentration suggested a stronger responsiveness of HM and nutrient transporters to changes in Zn concentration. The results of the PCA indicated that nutrient-related transporters were clustered together, but separately in AMF- and NM-treatment groups, at different Zn concentrations, indicating that AMF symbiosis may improves the metal stress resistance of plants mainly through nutrient regulation (Fig. 7).

## Conclusions

In the presence of symbiosis with AMF, we explored the variation in the transcription levels of six HM-related gene families under high-Zn conditions. The results indicated that the expression levels of one ZNT gene, three YSL genes, and one COPT gene were significantly upregulated, while those of one ZIFL gene and one Ctr gene were significantly downregulated (p < 0.05).

Under high-Zn conditions, genes related to the absorption of nutrients, mainly N, P, and K, were analyzed. We found that the expression levels of two NRT2 transporter genes, and one HAK transporter gene were significantly downregulated (p < 0.05). There was no significant upregulation of nutrient-related genes, indicating that a high Zn concentration inhibits the growth and development of plants. Meanwhile, under AMF symbiosis and high-Zn conditions, the expression levels of seven PHT genes, one NRT1 gene, two NRT2 genes, one HAK gene, and three AMT genes were significantly upregulated (p < 0.05), whereas those of only one PHT gene and one NRT1 gene were significantly downregulated (p < 0.05) (Fig. 8).

Here, we showed that AMF increases the resistance of *E. grandis* to high-Zn stress by improving the absorption of nutrients by the plant and regulating Zn uptake at the gene transcription level. Among these genes, 41 heavy metal transporters (ZNT:15, YSL:11, COPT/Ctr:8, CE/MTP:3, ZIFL:4) and 36 nutrition-related transporters are involved in Zn tolerance in *E. grandis* with AMF symbiosis. Although these genes' function was inferred from their homologous genes' function in model herbaceous plants, these candidate genes' multiple function still need to be investigated at whole plant and tissue level for the woody perennial plants having unique physiological and anatomical structure. Furthermore, the interaction between HM tolerance and nutrient acquisition should be furtherly explored to facilitate the genetic improvement of nutrient utilization in *E. grandis* at the HM-contaminated areas.

## Materials and methods

**Plant cultivation, inoculation, and Zn treatment**

The seedlings needed for the experiment were taken from *E. grandis* clone GL1, kindly provided by Dr. Chunjie Fan from the Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou, China. The AMF species *Rhizophagus irregularis* (BGCBJ09) was purchased from the Beijing Academy of Agriculture and Forestry Sciences (Beijing, China). The AMF was propagated using *Trifolium*.
Fig. 7 (See legend on previous page.)
repens, and the inoculum was collected to obtain spores. The AMF spores were obtained by sucrose-gradient centrifugation [95]. Uniform *E. grandis* seedlings were selected and transplanted into plastic pots (9 cm high, 10 cm diameter) with sterile medium (quartz sand:vermiculite, V:V = 2:1). Seedlings receiving inoculation treatment (hereafter referred to as the AMF-treatment group) were inoculated with 600 spores, which were added to the medium in each pot. Seedlings in the non-inoculation group were not inoculated with AMF spores (referred to as the NM-treatment group).

Plants were grown in a growth chamber with 25/20°C day/night temperatures, 60% relative humidity, and a 16/8-h light/dark photoperiod. To generate highly colonized roots, for 45 days post-inoculation (dpi), the plants were supplied with 50 mL of Long-Ashton solution [96] with a modified monosodium phosphate (NaH$_2$PO$_4$) concentration (30 μM) every 3 days. This solution contained 0.5 μM Zn$^{2+}$. At 46 day, each pot was immersed in a beaker filled with distilled water. The water was changed five times, once every 2h, to remove residual nutrients from the substrates. The AMF- and NM-treatment groups were then divided into three subgroups: low Zn concentration (0.01 μM Zn supplied as ZnCl$_2$), normal Zn concentration (0.5 μM Zn supplied as ZnCl$_2$), and high Zn concentration (150 μM Zn supplied as ZnCl$_2$). Zinc treatment were supplied with 50 mL of Long-Ashton solution with different Zn concentration every three days for fifteen days, five times in total. There were six pots (replicates) for each Zn treatment.

**Arbuscular mycorrhizal colonization rate measurement**

Mycorrhizal-colonized roots were stained with Wheat germ agglutinin-conjugated Alexa Fluor®488 (WGA-AF488) as described previously [97]. Briefly, harvested roots were placed in 50% ethanol for more than 4 h and then transferred to 20% (w/v) KOH for 2–3 days, followed by 0.1 M HCl for 2 h at room temperature. After removing the HCl, the samples were rinsed twice with distilled water and once with phosphate-buffered saline (PBS, pH 7.4), and then immersed in PBS/WGA-Alexa Fluor 488 staining solution (0.2 μg/mL) in the dark for more than 6 h at 37°C. The arbuscular mycorrhizal colonization rate was determined under a fluorescence microscope (NIKON, Eclipse Ni-U) using the gridline intersect method described by Giovannetti and Mosses (2010).

**Acid digestion and determination of Zn and inorganic phosphate (Pi) content**

Dried leaves and roots were ground and digested (50 mg) for 8 h in 5 mL of 6 M nitric acid (HNO$_3$) in closed-glass tubes on heating blocks at 90°C [98]. Extracts were diluted in a solution of 2% HNO$_3$. Pi and Zn concentrations were determined by inductively coupled plasma mass spectrometry (Thermo Scientific™...
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iCAP™ Q ICP-MS; Thermo Fisher Scientific, Waltham, MA, USA).

cDNA library construction and sequencing
Total RNA was extracted from individual samples using the RNAprep Pure Plant Kit (Tiangen, Beijing, China). For roots from each subgroup, three independent biological replicates were analyzed. RNA quality and concentration were determined by 1% agarose gel electrophoresis and spectrophotometry, respectively. mRNA was purified from total RNA using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) with oligo-dT magnetic beads. mRNA samples were fragmented into 150-bp fragments using a chemical reagent at high temperature. Double-stranded cDNA was synthesized using random hexamer primers and end-repaired with an exonuclease and a polymerase. The cDNA fragments and sequencing adapters were ligated with T4 DNA ligase (Thermo Scientific), according to the manufacturer’s protocol, and sequenced using an Illumina HiSeq™ 2000 sequencing system.

Transcriptome assembly
Raw data (raw reads) were first processed using in-house Perl scripts. Clean data (clean reads) were obtained by removing reads containing adapter sequences, poly-Ns, and low-quality reads from the raw data. Meanwhile, the number of bases scoring Q20 and Q30, the GC content, and the sequence duplication level of the clean data were calculated. All downstream analyses were based on clean data of high quality. Transcriptome assembly was accomplished using Trinity with min_kmer_cov set to 2 and all other parameters set to default values [99].

Gene annotation
Gene functional annotation was performed by sequence comparison with public databases. The Basic Local Alignment Search Tool was used to search for homology (E value <0.00001) between unique sequences and JGI nonredundant proteins (https://phytozome.jgi.doe.gov/pz/portal.html#!/info?alias=Org_Egrandinis), and Swiss-Prot (http://www.expasy.ch/sprot) databases were searched for protein sequence analysis. The best-hit transcripts were selected as unigenes. The Blast2GO program was then used to obtain Gene Ontology (GO) annotations and functional classification of all unigenes [100]. Enzyme Commission terms and biochemical pathway information were generated using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) (http://egg nog.embl.de/) was used to predict and classify potential functions based on known orthologous gene products [101].

Gene expression, signaling pathway analysis, and differential gene identification
Based on the number of reads mapping to a particular gene, the reads per kilobase per million reads (RPKM) metric was used to estimate the transcript levels of the genes [102]. Briefly, the RPKM value was calculated by dividing the number of reads mapped to each gene by the length of the gene and the number of reads from the library to compensate for slightly different read depths for different samples. An RPKM threshold value of 0.1 was set to detect the presence of a unigene, which corresponds to a false discovery rate (FDR) of 5% [103].

The DESeq program (http://www.huber.embl.de/users/anders/DESeq/) was used for the statistical analysis of differentially expressed genes (DEGs) between two samples [104]. DEGs were identified according to a difference in expression > two-fold and a significant p-value (padj <0.05), after adjusting for the FDR due to multiple testing procedures to minimize the chance of a type I error [105]. GO and KEGG analyses were also used to evaluate DEGs in a variety of biological pathways. Based on information from Nr (Non-Redundant Protein Sequence Database), eggNOG, GO, and KEGG analyses, DEGs involved in metamorphosis, immunity, and sensory perception were further investigated manually. The KEGG metabolic pathways for all unigenes with GO terms were constructed using an online tool with different colors to indicate different gene expression levels (http://www.genome.jp/kegg/tool/map_pathway2.html). Functional domain analysis was performed using ExPasy PROSITE (http://www.expasy.ch/tools/scanprosite/).

Data analyses
Gene families related to the transport and distribution of metals and nutrients in plants were selected for further analysis. These included zinc transporter (ZNT), copper transporter (COPT), zinc-induced facilitator (ZIF), yellow stripe-like (YSL), heavy metal ATPase (HMA), cation efflux (CE), phosphate transporter (PHT), ammonium transporter (AMT), nitrate transporter (NRT), and potassium transporter gene families. A cluster analysis of the transcriptional levels of these gene families was performed using TBtools (gitub.com/CJ-chen/TBtools). Phylogenetic analyses of each gene family were performed using the neighbor-joining method implemented in MEGA 6 (www.megasoftware.net). For principal component analysis (PCA), data were
standardized and computed using Origin2021b (https://www.originlab.com/). Pearson’s correlation analyses were performed using SPSS (IBM, Armonk, NY, USA).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03456-5.

Additional file 1: Table S1. General information of ZNT transporter genes identified in Eucalyptus grandis. Table S2. Pearson's correlation analysis on ZNT family genes. Table S3. Pearson's correlation analysis on COPT/Ctr family genes. Table S4. Pearson's correlation analysis on YSL family genes. Table S5. Pearson's correlation analysis on ZIFL family genes. Table S6. Pearson's correlation analysis on HMA family genes. Table S7. Pearson's correlation analysis on CE/MTP family genes. Table S8. Pearson's correlation analysis on PHT family genes. Table S9. Pearson's correlation analysis on AMT and NRT family genes. Table S10. Pearson's correlation analysis on Potassium transporters family genes.

Additional file 2. Additional file 3. Additional file 4. Additional file 5.

Acknowledgements
We thank Dr. Chunjie Fan (Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou, China) for providing the Eucalyptus material, Ping Luo for technical assistance, and we are very grateful to Yuqiao Su and Wenzhen Lai for their helpful suggestions and their valuable comments on the early manuscript.

Authors' contributions
X W, W H and M T conceived and designed the paper; X W, J L, Z L, L L collected and analyzed the literature; J L and Y K drafted the paper and prepared the figures; X X and H C, have revised the manuscript. All authors havepared the figures; X X and H C, have revised the manuscript. All authors have

Funding
This study was financially supported by the Laboratory of Lingnan Modern Agriculture Project, grant number (NZ2021025), the Key Projects of Guangzhou Science and Technology Plan (201904020022) and the National Natural Science Foundation of China (32001289).

Availability of data and materials
The datasets supporting the conclusion of this article are included in the article and its additional files, all gene expression data were deposited in Box (https://app.box.com/s/e2a12z0u7eekdpk8ncox4ai3h6fcpn9245ss) and Galaxy Project (https://usegalaxy.org/datasets/bbd44e69cb89d656b8c01481728cd72/display?to_ext=tabular) and Eucalyptus grandis reference genome from Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#info?alias=Org_Egrandis), as well as can be requested from HNT (hwz@scau.edu.cn).

Declarations
Ethics approval and consent to participate
The data collection of plants and fungi were carried out with permission of related institution, and complied with national or international guidelines and legislation.

Consent for publication
Not applicable as the manuscript contains no individual identifying data.

Competing interests
The authors declare that they have no competing interests.

Received: 7 September 2021   Accepted: 4 February 2022

Published online: 22 February 2022

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