Model systems to unravel the molecular mechanisms of heavy metal tolerance in the ericoid mycorrhizal symbiosis

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32	Abstract	Ericoid mycorrhizal plants dominate in harsh environments where nutrient-poor, acidic soil conditions result in a higher availability of potentially toxic metals. Although metal-tolerant plant species and ecotypes are known in the Ericaceae, metal tolerance in these plants has been mainly attributed to their association with ericoid mycorrhizal fungi. The mechanisms underlying plant protection by the fungal symbiont are poorly understood, whereas some insights have been achieved regarding the molecular mechanisms of heavy metal tolerance in the fungal symbiont. This review will briefly introduce the general features of heavy metal tolerance in mycorrhizal fungi and will then focus on the use of "omics" approaches and heterologous expression in model organisms to reveal the molecular bases of fungal response to heavy metals. Functional complementation in *Saccharomyces cerevisiae* has allowed the identification of several ericoid mycorrhizal fungi genes (i.e., antioxidant enzymes, metal transporters, and DNA damage repair proteins) that may contribute to metal tolerance in a metal-tolerant ericoid *Oidiodendron maius* isolate. Although a powerful system, the use of the yeast complementation assay to study metal tolerance in mycorrhizal symbioses has limitations. Thus, *O. maius* has been developed as a model system to study heavy metal tolerance mechanisms in mycorrhizal fungi, thanks to its high metal tolerance, easy handling and in vitro mycorrhization, stable genetic transformation, genomics, and transcriptomic and proteomic resources.
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REVIEW

Model systems to unravel the molecular mechanisms of heavy metal tolerance in the ericoid mycorrhizal symbiosis

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Abstract Ericoid mycorrhizal plants dominate in harsh environments where nutrient-poor, acidic soil conditions result in a higher availability of potentially toxic metals. Although metal-tolerant plant species and ecotypes are known in the Ericaceae, metal tolerance in these plants has been mainly attributed to their association with ericoid mycorrhizal fungi. The mechanisms underlying plant protection by the fungal symbiont are poorly understood, whereas some insights have been achieved regarding the molecular mechanisms of heavy metal tolerance in the fungal symbiont. This review will briefly introduce the general features of heavy metal tolerance in mycorrhizal fungi and will then focus on the use of "omics" approaches and heterologous expression in model organisms to reveal the molecular bases of fungal response to heavy metals. Functional complementation in *Saccharomyces cerevisiae* has allowed the identification of several ericoid mycorrhizal fungi genes (i.e., antioxidant enzymes, metal transporters, and DNA damage repair proteins) that may contribute to metal tolerance in a metal-tolerant ericoid *Oidiodendron maius* isolate. Although a powerful system, the use of the yeast complementation assay to study metal tolerance in mycorrhizal symbioses has limitations. Thus, *O. maius* has been developed as a model system to study heavy metal tolerance mechanisms in mycorrhizal fungi, thanks to its high metal tolerance, easy handling and in vitro mycorrhization, stable genetic transformation, genomics, and transcriptomic and proteomic resources.

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Keywords Ericoid mycorrhizal fungi · Metal tolerance · Yeast model system · Omics approaches

Mycorrhizal fungi protect their host plant from abiotic stress, including heavy metals

Symbioses between plants and beneficial soil microorganisms, such as mycorrhizal fungi, promote plant growth by improving plant nutrition and competition, but they also help plants to cope with several environmental stresses (Jung et al. 2012). For example, it has been documented by several authors that both ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi can improve drought tolerance and enhance salt tolerance of their host plants (Luo et al. 2011, 2014; Ma et al. 2014; Talaat and Shawky 2014). Heavy metals are an important source of environmental stress because they can be very toxic at above threshold concentrations. Metal-adapted plant species or ecotypes survive in metal-contaminated environments mainly thanks to exclusion or detoxification mechanisms (see e.g., Hall 2002; Ernst 2006; Verbruggen et al. 2009). However, plants can also achieve metal tolerance through the association with mycorrhizal fungi. In fact, in spite of some variations in metal accumulation in the host plant, most studies indicate that ECM and AM plants accumulate less metal inside their tissues and grow better than non-mycorrhizal plants do when exposed to an excess of heavy metals (Adriaensen et al. 2004, 2005, 2006; Audet and Charest 2006; Jourand et al. 2010; Walker et al. 2004). In addition to protecting the plant from excess uptake, mycorrhizal fungi may also enhance plant internal detoxification (Luo et al. 2014).

Plants in the family Ericaceae dominate in nutrient-poor and stressful soil conditions. Metal-tolerant species and ecotypes have been found also in these plants and suggest specific

adaptation mechanisms (Sharples et al. 2000a; Rossini-Oliva et al. 2012). However, more important in these soil conditions seems to be the association of Ericaceae with ericoid mycorrhizal (ERM) fungi, which form intracellular symbioses in their fine hair roots (Fig. 1). Metal tolerance in ERM plants has been linked to the stress tolerance of their fungal partners, which would increase host-plant tolerance as well (Bradley et al. 1981, 1982; Cairney and Meharg 2003). Soils colonized by Ericaceae are generally acidic, and the low pH and anaerobic soil conditions facilitate mobilization of heavy metal ions (Meharg and Cairney 2000). Bradley et al. (1981, 1982) demonstrated for the first time the importance of ERM fungi in increasing resistance of *Calluna vulgaris* to heavy metals, and other authors later described metal tolerance in ERM fungal isolates from sites with different pollution (Martino et al. 2000a; Sharples et al. 2000b; Vallino et al. 2011). Despite these observations, our understanding of the mechanisms underlying plant protection by the ERM fungi is still poor, whereas increasing knowledge is being gathered on the mechanisms of heavy metal tolerance in ERM fungi. In particular, a number of mechanisms has been identified in metal-tolerant isolates of the ERM fungus *Oidiodendron maius*, a species in the Leotiomycetes (Ascomycetes) isolated from experimental plots in the Niepolomice Forest (Poland), a site heavily contaminated with industrial dusts and containing high concentrations of Zn, Cd, and Al (Martino et al. 2000a, 2000b, 2002, 2003; Vallino et al. 2005, 2009; Abbà et al. 2011; Khouja et al. 2013, 2014).

Starting from a brief summary of the general features of heavy metal tolerance in mycorrhizal fungi, this review will focus on the use of yeast, a well-established fungal model system, to identify genes involved in heavy metal tolerance in fungi. Thanks to functional complementation of *Saccharomyces cerevisiae* metal-sensitive mutants, several genes that may contribute to metal tolerance were identified in a heavy metal-tolerant isolate of the ERM species *Oidiodendron maius*. We will also describe the features that helped us to develop *O. maius* as a model system for ERM fungi and some recent findings on the mechanisms of heavy metal tolerance in this species.

Mechanisms of heavy metal detoxification in mycorrhizal fungi

Metal elements are directly or indirectly involved in all aspects of microbial growth (Gadd 1993, 2010), with several of them playing essential functions (e.g., Zn, Cu, Mg, Fe) and some (e.g., Cs, Al, Cd, Hg, and Pb) having no known function in most organisms and being therefore already toxic at low concentrations. In addition, heavy metals often influence the uptake and concentrations of essential elements such as phosphorus and nitrogen (Krznaric et al. 2009; Luo et al. 2014). Molecular recognition allows organisms to differentiate between essential and non-essential elements and, if necessary, to partition them in different ways. The toxicity of heavy metals to both mycorrhizal fungi and their host plants can result from molecular disfunctions caused by the displacement of essential metals in biomolecules (e.g., enzymes and transcription factors), from the binding of metals to thiol groups, which inhibits functions of the target biomolecules, and from overproduction of ROS as the consequence of blocked thiol groups (Sharma and Dietz 2009; Schützendübel and Polle 2002).

Emerging evidence suggests that the cellular mechanisms involved in detoxification of excess heavy metals by mycorrhizal fungi include (similar to other fungi): (a) the biosorption of metals to the fungal cell walls, (b) the binding of heavy

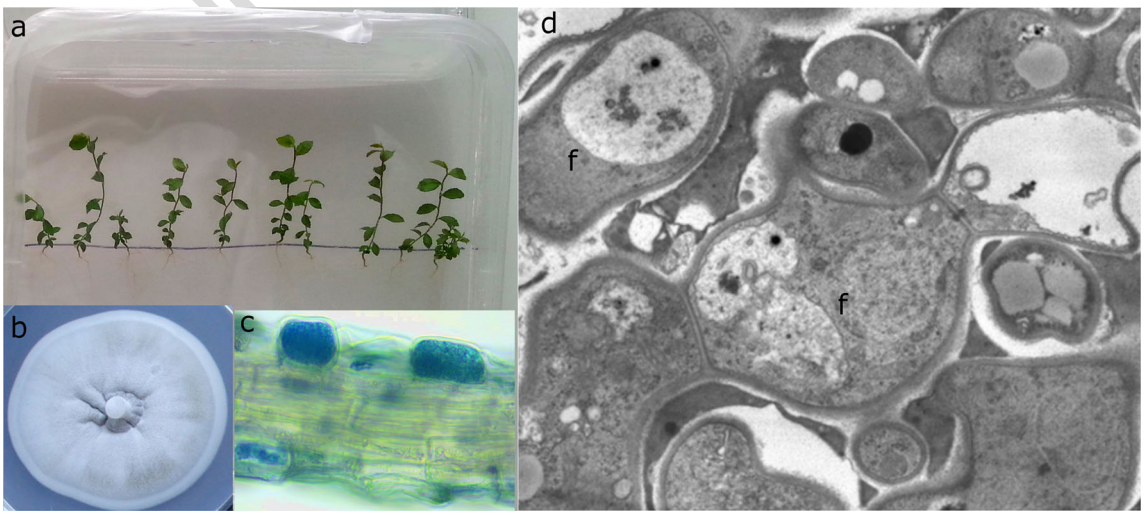

Fig. 1 General features of ericoid mycorrhiza. **a** Cocultivation of *Vaccinium myrtillus* plantlets with *Oidiodendron maius* Zn, generating mycorrhizal plants; **b** *O. maius* Zn mycelium grown on Czapek-dox agar medium; **c** *V. myrtillus* root with hyphal coils of *O. maius* Zn (cotton blue staining); **d** TEM section of a mycorrhizal *V. myrtillus* root cell: *f* indicates the fungal hyphae

metals to extracellular exudates and consequent possible precipitation, (c) the decreased uptake and/or removal of metal ions from the cytosol via transporters located at the plasma membrane, (d) the chelation of metal ions in the cytosol by compounds such as glutathione, metallothioneins and, rarely, phytochelatins, (e) the compartmentation of metals in the vacuole or other subcellular structures, (f) the repair of metal-damaged biomolecules, and (g) antioxidative mechanisms that allow the fungus to directly or indirectly counteract accumulation of ROS and oxidative stress (Bellion et al. 2006; Colpaert et al. 2011; Gallego et al. 2012; Seth et al. 2012). Some of these mechanisms are constitutively present, whereas others are only activated when metals exceed a threshold value (Colpaert et al. 2011).

Yeast as a model system to study fungal response to heavy metals

Model systems are important tools to unravel the molecular mechanisms underlying biological processes. *S. cerevisiae* in particular is an attractive model organism due to the fact that it is very easy to maintain in the lab and has a fast life cycle. In addition, its genome has been fully sequenced (Goffeau et al. 1996), thus making genetic manipulation easier and analyses based on high throughput approaches (i.e., "omics" approaches such as genomics, transcriptomics, proteomics, metabolomics, and phenomics) more informative than in other organisms.

The most straightforward "omics" approaches to investigate cellular responses to heavy metal exposure in *S. cerevisiae* have been proteomics (Hu et al. 2003; Vido et al. 2001) and transcriptomics (Hosiner et al. 2014). Although mainly descriptive, both approaches have provided useful information on the influence of heavy metals on gene and protein expression. For example, a recent trascriptomic experiment showed that the acute (30 min) metal stress by Ag, Al, As, Cd, Co, Hg, Mn, Ni, V, and Zn induces differential expression in about 15 % of the yeast transcripts, with some common processes being activated by distinct groups of metals, but also unique expression patterns for particular metals (Hosiner et al. 2014).

Interesting results have been also derived from deletomics, i.e., the analysis of a deletion mutant collection covering nearly the entire yeast genome. A nearly complete set (96 % of all annotated ORFs) of gene-disrupted mutants was obtained in *S. cerevisiae* by Giaever et al. (2002). The phenotypic consequence of gene loss in individual yeast mutants (e.g., increase or decreased growth upon metal exposure) can in fact lead to the identification of the metabolic pathways involved.

The screening of the yeast deletion mutant collection to assess the role of non-essential genes in the response to heavy metals (Zn, Cd, Hg, Cu, Ag, Cr, As and Ni) revealed a major role of the vacuole for metal sequestration and detoxification. A wide range of additional cellular functions likely involved in general stress response and repair of damage caused by metals were also identified, such as the GSH and reduced sulfur metabolism, metal chelation, antioxidant defense, protein turnover, mRNA decay and trafficking, structural and functional integrity of the membranes, and DNA repair. The chemical properties of the metals likely define the responsive genes and the cell toxicity effects. For example, it is not surprising that Cd and Hg raised similar responses because they share a similar thiophilicity and lack of redox activity, as well as Mn, Ni, Zn, and Co, that are all non-redox transition metals, whereas Fe(III) is redox-active and was the most divergent metal investigated (Jin et al. 2008; Ruotolo et al. 2008). In addition, Ruotolo et al. (2008) suggested that components of the high-affinity Fe transport pathway contributed to the yeast tolerance to Cu, Mn, Ni, Co, and Zn, but not to Fe, suggesting that Fe homeostasis requires different mechanisms. Bleackley et al. (2011) suggested a lack of metal-specificity based on the results of a new deletome screening showing that Mn, Ni, Zn, and Co sensitivity was common to a number of deletion strains. These authors discussed that promiscuity in metal binding in proteins likely preceded metal binding specificity during evolution, and the overlap in tolerance pathways may be interpreted as a relic of metal binding promiscuity. However, most of the vacuolar deletion strains were sensitive to Mn, Zn, Ni, and Co (all of which are stored in the vacuole), but not to Fe.

Yeast functional complementation to identify ERM fungal genes involved in heavy metal tolerance

Omics approaches in yeast have been instrumental to investigate fungal responses to heavy metals, and they have unraveled common as well as metal-specific pathways. However, *S. cerevisiae* is not very tolerant to heavy metals, and whereas its deletome/transcriptome can help to explain stress response of fungi when exposed to heavy metals, it has limitations to unravel mechanisms underlying heavy metal tolerance in metal-tolerant filamentous fungi. Other methodologies, employing the yeast model system for heterologous expression and functional complementation of deletion mutants, have been helpful in identifying genes from metal-tolerant ECM and AM mycorrhizal fungi (Courbot et al. 2004; González-Guerrero et al. 2005; Lanfranco et al. 2002). Functional complementation of yeast mutations was used for the first time in ERM fungi as a targeted approach to demonstrate the role of Cu/Zn superoxide dismutase (SOD) enzyme in metal tolerance (Vallino et al. 2009). The synthesis of antioxidant enzymes such as catalases, peroxidases, and SODs is known to protect fungi from the oxidative stress caused by heavy metals (Guelfi et al. 2003; Jacob et al. 2001; Todorova

et al. 2008). In particular, SODs play a protective role against free superoxide radical toxicity (Fridovich 1995) and their induction by heavy metals has been described in plants, animals, and microorganisms (Chongpraditnun et al. 1992; Yoo et al. 1999; Vido et al. 2001). Exposure of *O. maius* Zn, a metal-tolerant isolate derived from the Niepolomice (Poland) contaminated soil, to Zn and Cd increased the amount and activity of both intracellular and extracellular SOD enzymes that could help both the ERM fungus and the host plant to cope with ROS formation (Chiapello et al. 2015; Martino et al. 2002; Vallino et al. 2009). As these enzymes are metalloenzymes, like most oxidoreductases, the increased production of an extracellular Cu/Zn SOD in *O. maius* Zn may also reduce metal toxicity thanks to its metal binding capacity (Vallino et al. 2009). A metal-sensitive yeast mutant lacking this enzyme regained metal tolerance to Zn, Cu, and Cd when transformed with the *O. maius* Zn full-length cDNA coding for this enzyme.

Targeted approaches, such as yeast functional complementation with the *OmSod1* gene, normally rely on existing knowledge and may be helpful confirming the role of individual components in heavy metal tolerance, but they would miss so far unidentified mechanisms that can be better addressed by untargeted approaches. Untargeted approaches are in fact an important source of novel information, especially if they are supported by functional assays (Ruytinx et al. 2011). Metal-sensitive yeast mutants have been used to screen by functional complementation whole cDNA libraries from mycorrhizal fungi (Leonhardt et al. 2014a; Osobová et al. 2011; Ramesh et al. 2009) in order to identify genes capable of conferring to the transformants the ability to grow in metal-containing media. The same approach has lead, in the metal-tolerant ERM fungus *O. maius* Zn, to the identification of some metal transporters (Khouja et al. 2013), but also to the discovery of the novel protein OmFCR, a member of the PLAC8-domain containing proteins, likely involved in DNA damage repair (Abbà et al. 2011). These genes are described in the following paragraphs.

O. maius Zn gene coding for metal transporters

Membrane transporters can reduce heavy metal toxicity because they can regulate cytoplasmic metal concentrations either by limiting metal uptake or by increasing metal efflux and/or compartmentation in cell organelles (Pócsi 2011). In order to identify membrane transporters involved in heavy metal tolerance in *O. maius* Zn, functional screening of a cDNA library obtained from this ERM fungus growing on Cd-amended medium was performed in the zinc-sensitive Δ*zrc1* mutant of *S. cerevisiae*. Two full-length cDNAs were isolated and further characterized in yeast, respectively encoding OmZnT1, a member of the cation diffusion facilitator family of zinc transporters, and OmFET, a member of the iron permease family (Khouja et al. 2013; Fig. 2b).

Zn homeostasis has been largely investigated in yeast, whereas much less is known in filamentous fungi. In mycorrhizal fungi, although many putative Zn-transporter genes have been identified *in silico* (e.g., Tamayo et al. 2014), only a few have been functionally characterized in yeast: the *RaZIP1* and *RaZIP2* from *Russula atropurpurea* (Leonhardt et al., 2014b), *HcZnt1* from *Hebeloma cylindrosporum* (Blaudez and Chalot 2011), *GiZnT1* from *Rizophagus irregularis* (renamed *RiZnT1*; González-Guerrero et al. 2005), and *OmZnT1* and *OmFET* from *O. maius* Zn (Khouja et al. 2013). In yeast, OmZnT1 was located in the ER membrane and was able to restore growth of Zn and Co sensitive mutants lacking vacuolar transporters, suggesting that it could detoxify zinc by delivery and compartimentation into the ER, a common strategy of metal tolerance. Similarly, the ER-resident CDF proteins ZHF1 from the yeast *Schizosaccharomyces pombe* (Clemens et al. 2002) and HcZnT1 from the basidiomycete *Hebeloma cylindrosporum* (Blaudez and Chalot 2011) have been demonstrated to confer zinc tolerance in yeast. The release of the *O. maius* Zn genome sequence (http://genome.jgi.doe.gov/; Kohler et al. 2015) revealed the presence of two other putative Zn-CDF transporters in this organism.

OmFET is a low-affinity iron transporter that has also been found in other filamentous fungi, but it has been fully characterized only in *S. cerevisiae* (Kosman 2003), showing relatively low substrate specificity. Measurement of intracellular ion concentration indicates that yeast transformants constitutively expressing OmFET contained significantly less Zn than cells did harboring the empty vector, which would explain the positive selection of *OmFET* during the screening of the *O. maius* cDNA library (Khouja et al. 2013). Although this transporter belongs to the iron permeases family, we could not find significant iron accumulation in the OmFET-expressing yeast, as compared to control cells, while the magnesium content was always significantly higher in OmFET-expressing cells than in control cells. It was thus suggested that OmFET could enhance zinc tolerance in yeast by increasing the cellular content of magnesium, which has a general protective effect against different heavy metal cations such as manganese, copper, nickel, cadmium, and cobalt in yeast (Blackwell et al. 1998; Joho et al. 1991; Karamushka and Gadd 1994) and zinc and cadmium in plants (Kupper and Kochian, 2010; Pedler et al. 2004).

O. maius Zn gene coding for components of the DNA damage repair system

A novel gene conferring Cd resistance was isolated from a cDNA library obtained from *O. maius* Zn exposed to Cd by functional complementation of a metal-sensitive yeast mutant

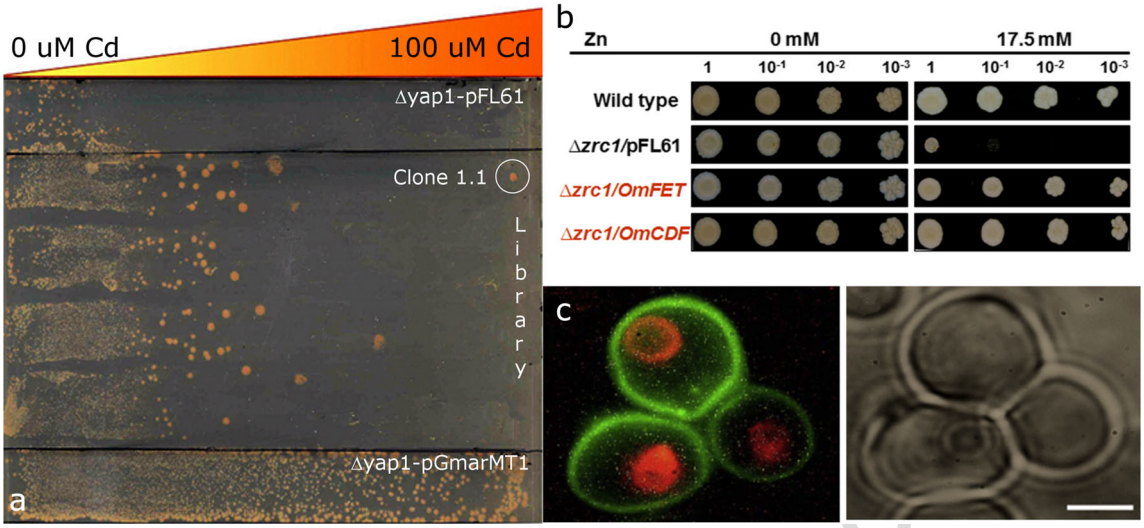

Fig 2 Heterologous expression in yeast of *O. maius* Zn genes. **a** The *yap1*-deficient yeast strain was transformed with a pFL61-cDNA library of *Oidiodendron maius* Zn exposed to $CdSO_4$ and, the yeast cells were spread on SD-agar plates containing a linear concentration gradient (0–100 mM) of $CdSO_4$: the clones growing at the highest concentrations were all expressing *OmFCR1*; **b** functional complementation of the metal-sensitive Δ*zrc1* yeast strains by *OmFET* and *OmZnT1*; **c** localization of OmFET/GFP on the yeast plasma membrane by epifluorescence microscopy, and corresponding bright field image

(Δ*yap1*). The new gene was called *O. maius* fungal cadmium resistance 1 (*OmFCR1*) because of the structural and functional similarities with its ortholog in *Arabidopsis thaliana* plant cadmium resistance (*AtPCR*). These genes both harbor a PLAC-8 (or DUF614) conserved domain whose function remains unknown despite a number of studies that attributed different roles to members of this protein family, ranging from the control of cell cycle and cell size in both animal and plants (Frary et al. 2000; Guo et al. 2010; Jimenez-preitner et al. 2011, 2012; Rogulski et al. 2005) to a function in cadmium resistance for AtPCR (Song et al. 2004). OmFCR1 is likely to confer Cd resistance by interacting with components of the mismatch repair (MMR) system involved in DNA damage repair (Abbà et al. 2011; Fig. 2a). More recently, another gene which also harbors a PLAC-8 domain was identified in the genome of *O. maius* Zn. This gene, called *OmFCR2*, was able to rescue the Cd-sensitive phenotype in mutant yeast, although less pronounced than *OmFCR1* (Di Vietro et al. 2014). Expression of *OmFCR1* in *O. maius* Zn, as measured by real-time qPCR, significantly increased after 24 h of Cd exposure, while the expression of *OmFCR2* was constant and generally lower than *OmFCR1* expression. Hence, these two genes share a similar function in Cd response but show a different expression trend, thus suggesting a possible modulation of the response to Cd, just like it would be expected for paralogs (Gabaldon and Koonin 2013). Besides, both *OmFCR1* and *OmFCR2* promoter regions harbor putative metal response elements (MRE), suggesting that the metal-mediated induction has been conserved after duplication (Di Vietro et al. 2014). The generation of *OmFCR1* knock-out mutants in *O. maius* Zn had not resulted in a Cd-sensitive phenotype, and a possible explanation is that OmFCR2 could contribute to the resistant phenotype in the *OmFCR1* knock-out mutants, together with a number of cellular/molecular responses activated by the fungus and described in other studies (Martino et al. 2000b, 2003; Khouja et al. 2013; Vallino et al. 2009).

O. maius as a model system for ericoid mycorrhizal fungi

Yeast has been very helpful in the identification of heterologous genes involved in metal tolerance from mycorrhizal fungi, by both targeted and untargeted functional complementation. However, the use of the yeast system has limitations for the study of metal tolerance mechanisms in mycorrhizal fungi because it mainly reveals mechanisms based on individual molecular components (e.g., metal transporters, antioxidant enzymes, etc.) rather than more complex cellular functions. In addition, some of the mechanisms that operate in mycorrhizal fungi may also confer protection to the host plant, a potential feature that could not be tested in yeast. Hence, the elucidation of the mechanisms of heavy metal homeostasis in mycorrhizal fungi and their possible roles in plant protection require the development of mycorrhizal model systems, possibly with characteristics of heavy metal tolerance, ease of laboratory handling, knowledge of the genome sequence, and availability of genetic transformation protocols. For ERM, *O. maius* Zn is emerging as a model system to investigate cellular processes related to heavy metal tolerance. This ascomycete can be easily grown in vitro, where it produces asexual conidia containing a single haploid nucleus, which can germinate to produce a homokaryotic mycelium. In addition to the haploid genome and easy culturing, tools have been developed for *O. maius* over the years, such as genetic

transformation and omics databases. This ERM fungus is also a relatively easy system to study the expression and function of fungal genes during mycorrhizal interactions (Kohler et al. 2015), but it will be also an interesting model system for the functional study of genes from other less genetically tractable mycorrhizal fungi by heterologous expression, and for the identification of common pathways in mycorrhizal interactions. For example, constitutive expression in *O. maius* Zn of an AM fungal gene induced during arbuscule development resulted in a higher percentage of *Vaccinium myrtillus* root colonization (Lanfranco et al. unpublished data).

Omics approaches to identify mechanisms of heavy metal tolerance in *O. maius* Zn

Large-scale experiments involving omics techniques are now routinely used in various research disciplines, including mycorrhizal research (Kohler et al. 2015; Laparre et al. 2014; Tisserant et al. 2012, 2013; Vincent et al. 2011), and some omics approaches have been recently applied also to ERM fungi. A first attempt to investigate ERM fungal genes involved in zinc tolerance through an untargeted approach was through the sequencing of a small EST collection (Vallino et al. 2005). By monitoring variation in gene expression after treatment with high Zn concentrations through reverse Northern blot hybridization, 16 unigenes were shown to be either up or downregulated. However, none of them corresponded to previously reported heavy metal responsive or stress-related genes. The fully sequenced genome of *O. maius* Zn and the availability of transcriptomic data need to be further exploited to understand the molecular mechanisms and cellular processes underlying heavy metal tolerance in ERM fungi.

Comparative high-throughput proteomics, another "omic" approach, was more recently applied to investigate protein accumulation in *O. maius* Zn exposed to zinc and cadmium (Chiapello et al. 2015). Cadmium selectively induced molecular chaperones of the Hsp90 family, cytoskeletal proteins, and components of the translation machinery, while zinc significantly upregulated metabolic pathways related to energy production and carbohydrate metabolism, suggesting that adaptation of this isolate to Zn exposure mainly involved the primary metabolism. Common proteins induced by the two metal ions were the antioxidant enzyme Cu/Zn SOD (further supporting earlier experiments by Abbà et al. 2009 and Vallino et al. 2009) and ubiquitin. The covalent attachment of ubiquitin to lysine residues of proteins is a post-translational modification originally described as a destruction tag that directs misfolded or disused proteins to the proteasome (Hall 2002). Some components of the proteasome were identified by 2-DE in the mycelium exposed to both cadmium and zinc suggesting the induction of the proteolytic activity eliminating ubiquitinated proteins as defence mechanism. Several proteins involved in ubiquitin-dependent proteolysis were also identified by Muller et al. (2007) when comparing the gene expression profiles of a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate exposed to increasing external zinc concentrations.

An interesting protein identified by shotgun proteomics and induced in *O. maius* Zn by both cadmium and zinc was the enzyme agmatinase, a key enzyme in the biosynthesis of polyamines (Dudkowska et al. 2003). Identification of agmatinase in the *O. maius* proteome in response to metals is very intriguing. Polyamines are positively charged small molecules found in prokaryotic and eukaryotic cells; putrescine and spermidine, in particular, are believed to occur in all living cells and to be implicated in many fundamental cellular processes (Igarashi and Kashiwagi 2000; Kusano et al. 2008). In plants, polyamine accumulation appears to be a universal response to stress, including toxic heavy metal concentrations (Alcázar et al. 2010; Minocha et al. 2014). In fungi, polyamines are essential to support growth and to regulate a wide variety of biological processes (Davis 1996; Valdés-Santiago et al. 2010), but little is known about their possible role in stress tolerance (Valdés-Santiago and Ruiz-Herrera 2014). Polyamine accumulation in response to heavy metals was investigated in the ECM fungus *Paxillus involutus*, where lead and zinc exposure specifically increased cellular concentrations of some polyamines (Zarb and Walters 1995; 1996). Although the role of polyamines in the ERM fungal response to environmental stress requires further investigations, the results by Chiapello et al. (2015) suggest that the biosynthetic pathway leading to their formation is induced by both zinc and cadmium in *O. maius* Zn.

Genetic transformation of *O. maius* Zn to identify mechanisms of metal tolerance

Further tools have been developed for *O. maius* Zn and include protocols for stable genetic transformation (Fig. 3) by both PEG- and *Agrobacterium*-mediated transformation (Martino et al. 2007; Abbà et al. 2009). Although several ECM fungi have been stably transformed (Combier et al. 2003; Kemppainen et al. 2005; Marmeisse et al. 1992; Pardo et al. 2002; Rodriguez-Tovar et al. 2005), *O. maius* Zn remains so far the only example of genetic transformation of an endomycorrhizal fungus. Genetic transformation of *O. maius* Zn has been used to disrupt gene functions in order to identify possible alterations in the fungal phenotype. The complete and stable inactivation of a target gene via homologous recombination at the wild-type locus (gene knock-out) is in fact the most direct way to explore gene function, and it was successfully applied in *O. maius* Zn for the first time among mycorrhizal fungi. The SOD1-null mutant showed an

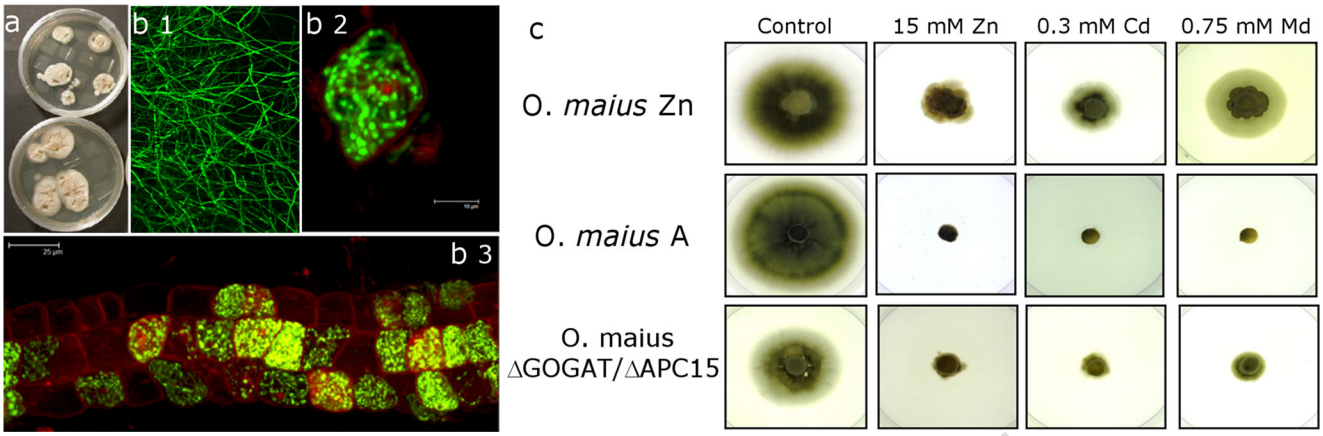

Fig 3 Genetic manipulation of *Oidiodendron maius* Zn. **a** *O. maius* Zn mutants selected for their hygromycin B resistance after *Agrobacterium tumefaciens*-mediated transformation; b. confocal microscopy images of *O. maius* Zn expressing EGFP (**b**.1 free living mycelium; **b**.2 hyphal coil; **b**.3 *Vaccinium myrtillus* colonized root); **c** oxidative stress tolerance assay of *O. maius* Zn (stress-tolerant WT isolate), *O. maius* A (stress-sensitive WT isolate), and the *O. maius* Δ*GOGAT*/Δ*APC15* strain (a stress-sensitive mutant obtained by random genetic transformation): the three strains were grown on media amended with 15 mM $ZnSO_4$, 0.3 mM $CdSO_4$, or 0.75 mM menadione

imbalanced ROS homeostasis as well as a decreased Cd and Zn tolerance and a decrease of the formation of mycorrhizal coils with respect to the wild-type (WT) strain. These results suggested that the ROS scavenging has an important role not only in the stress defence but also in the signaling between *O. maius* Zn and its host plant (Abbà et al. 2009).

In addition to the disruption of target genes by homologous recombination, genetic transformation can be used for random insertional mutagenesis. This approach has been used in *O. maius* Zn to build up a library of more than 2000 random mutants. This library was screened for sensitivity to heavy metals (Zn and Cd) and oxidative stress (menadione), and a number of mutants with altered phenotype and/or impaired growth in one or more of these conditions were selected. One of these mutants, in addition to an altered metal stress tolerance (Figs. 3c and 4), also showed impaired N-metabolism and was further characterized (Khouja et al. 2014). The glutamate synthase (GOGAT), a key enzyme in nitrogen metabolism, and its adjacent gene, *APC15*, were partially deleted. Genetic transformation was used to recomplement the disrupted *OmAPC15* gene with the functional *O. maius* Zn gene, and assays on two *OmAPC15*-recomplemented strains ascribed the metal sensitive phenotype to the deletion of the *OmGOGAT* gene. The *OmGOGAT*-deleted strain also showed a reduction of the glutamine synthetase (GS) activity and an upregulation of the alternative NADP-glutamate dehydrogenase pathway for glutamate biosynthesis, suggesting a strong alteration of the N-assimilation pathway. Unless they were supplemented with glutamine, *O. maius* Zn transformants lacking OmGOGAT were very sensitive to zinc. A number of studies in plants demonstrate the significance of nitrogen containing metabolites in the response to heavy metals (Sharma and Dietz 2006) and report in particular the involvement of glutamine and GS enzyme activity in the tolerance mechanisms to oxidative stress induced by metals (Hradilová et al. 2010; Ker and Charest, 2010; Kieffer et al. 2008; Wang et al. 2008). Glutamine synthetase was found to be upregulated by Cd in several plant species (Kieffer et al. 2008; Rana et al. 2008; Sarry et al. 2006; Wang et al. 2008) and was positively correlated with Cd tolerance in the hyperaccumulator *Noccaea caerulescens* (Tuomainen et al. 2006). Nitrogen uptake via glutamine/glutamate cycle is also linked to the glutathione biosynthesis (Li et al. 1993; Matés et al. 2002), and a possible role of glutathione is to reduce the concentration of free metal ions in the cell and prevent an increase in the production of reactive oxygen species under heavy metal stress (Xu et al. 2009). This could suggest that a possible reduced glutathione biosynthesis in the *OmGOGAT*-deleted strain would

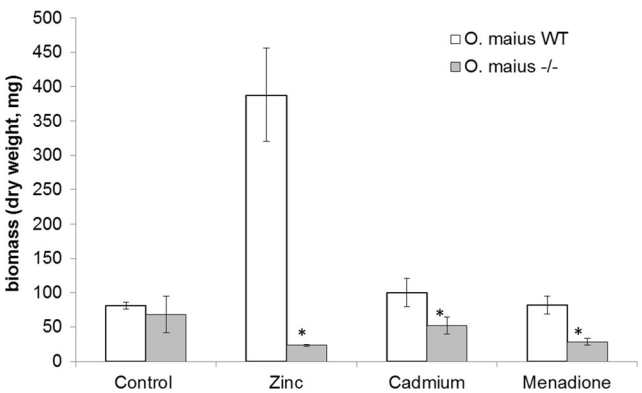

Fig. 4 Stress tolerance of the *Oidiodendron maius* Zn wild-type (white bars, *O. maius* WT) and the *O. maius* Δ*GOGAT*/Δ*APC15* strain (*light gray bars*, *O. maius* −/−). The two fungal strains were grown in liquid media supplemented or not (control) with 10 mM ZnSO4, 0.1 mM CdSO4 or 0.5 mM of menadione. The *bars* represent the average of three replicates, with standard deviation. *Asterisk* indicates significant differences between the *O. maius* WT and *O. maius* −/− strains

contribute to its stress-sensitive phenotype and to its selection in the random-mutant screening. Exogenously supplied glutamine could compensate the defect of glutamine biosynthesis, and the beneficial effect of glutamine was particularly evident when mycelia were exposed to zinc, as reported for plants (Hradilová et al. 2010; Rossini Oliva et al. 2012). These results by Khouja et al. (2014) demonstrate interplay between heavy metal tolerance and nitrogen metabolism and that some intermediate of nitrogen metabolism might be central to the fungal response to heavy metals.

Pythochelatins play an important role in metal tolerance in plants, and gene coding for pythochelatin synthase, or putative homologs of this enzyme, have been recently found in some fungal genomes (Bolchi 2011, Shine 2015). Phytochelatin synthase genes seem to be absent in *O. maius* Zn (unpublished data), making this fungus an interesting system for heterologous expression of this gene, in order to evaluate whether it confers higher metal tolerance and/or whether it affects the expression of other defense genes.

Perspectives for the study of metal tolerance in ERM fungi

As illustrated above, fungal model systems have been instrumental to identify some of the molecular components of heavy metal tolerance in ERM fungi. However, they have been used to investigate individual genes of single organisms (i.e., *O. maius* Zn) that could represent specific detoxification mechanisms. An interesting point will be to understand whether and how many of these tolerance mechanisms are the results of environment-driven adaptive evolution possibly found at the population level.

Heavy metal toxicity represents a strong selection pressure, and adaptation of ecto- and endomycorrhizal fungi to heavy metal soil pollution of anthropic origin is suggested by several studies (Adriaensen et al. 2005; Colpaert et al. 2004; Krznaric et al. 2009; Leyval et al. 1997; Meharg and Cairney 2000). Similarly, metal-tolerant ERM fungi with metal-specific tolerance mechanisms have been isolated from polluted sites. For example, Sharples et al. (2001) isolated As-tolerant strains of *Rhizoscyphus ericae* from the roots of *C. vulgaris* collected in an As/Cu-contaminated mine. These fungi were able to specifically transport arsenate out of the hyphae, thus representing a barrier for arsenate uptake into the plant. Similarly, increased zinc efflux was found to be an adaptive mechanism of zinc tolerance in isolates of the ECM *Suillus bovinus* collected from soils heavily contaminated with this metal (Ruytinx et al. 2013).

Some indications of adaptive metal tolerance were also reported for *O. maius* by Vallino et al. (2011), who investigated a number of fungal isolates derived from soils with different pollutants, namely a serpentine site enriched in Cr and Ni, an industrial soil mainly contaminated with Cd and Zn, and a non-polluted soil. These *O. maius* isolates showed a statistically significant difference in their ability to grow in the presence of the metal contaminants typical of the site of origin. The isolates more tolerant to Cr and Ni were those originated from the serpentine site, while the isolates more tolerant to Zn and Cd were those from the industrially polluted site enriched in these contaminants (Vallino et al. 2011). Some genetic features were further investigated in these isolates. In particular, DNA mutation rate (in terms of base substitution and insertion/deletions) was assessed for specific regions of the fungal genome that have different significance in metal tolerance: the "functional" gene coding for the Cu/Zn SOD, already demonstrated to play a role in metal tolerance (Vallino et al. 2009), and the "neutral" ribosomal ITS gene. *O. maius* isolates from all sites, polluted and non-polluted, showed higher mutation rates in the functional *Sod1* locus, important for fungal survival, than in the neutral ITS locus (Vallino et al. 2011). In addition, *O. maius* isolates from heavily polluted industrial soils showed a significantly higher mutation rates in the *Sod1* locus than fungi from less polluted or non-polluted sites. The accumulation of mutations was not the result of a random process because a higher mutation rate was calculated for the *Sod1* promoters of metal-exposed than non-exposed isolates, while the mutation rate was similar when the coding sequence was considered (Vallino et al. 2011). Although limited to a single locus and to a small population of ERM fungi, these observations would indicate that mutagenesis induced by environmental stress may target specific gene regions and suggest a rapid evolution of key pathways, like stress signaling, driven by the need of defense of the organism (Nikolaou et al. 2009).

It will be therefore interesting to extend these types of analyses to whole genomes. Sequencing of fungal genomes is becoming relatively simple and cost-effective and, based on the already fully sequenced genome of *O. maius* Zn, resequencing of several other *O. maius* isolates from metal-tolerant and metal-sensitive populations should provide additional information on the evolution of metal tolerance molecular mechanisms.

Conclusions

Metal-tolerant ERM fungal isolates can successfully colonize heavy metal-polluted soils and protect their host plants from metal toxicity through mechanisms that are still largely unknown. By contrast, a combination of targeted and untargeted approaches together with the use of yeast as a model system for heterologous gene expression have helped us to unravel some of the mechanisms underlying ERM fungal metal tolerance. An emerging model system for ERM fungi is *O. maius*, a species for which metal-sensitive and metal-tolerant isolates are available. Genome, transcriptome, and proteome

databases, as well as genetic tools, have been developed for the metal-tolerant isolate *O. maius* Zn, and they have been instrumental in identifying not only tolerance mechanisms already known from other organisms but also novel molecular components and metabolic pathways involved in metal tolerance. Multiple mechanisms likely enable metal-tolerant ERM fungi to protect themselves and their host plant from toxic compounds. Further use of omics approaches is already in progress to compare metal-tolerant and metal-sensitive ERM isolates, as well as transcriptomic analyses of the host plant under different conditions. These data will help not only in dissecting the molecular and cellular pathways involved in heavy metal tolerance in ERM fungi, but hopefully also in revealing the mechanisms underlying mycorrhiza-modulated host plant tolerance to heavy metals.

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Q2. "Bolchi 2011" is cited in text but not given in the reference list. Please provide details in the list or delete the citation from the text.
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