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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.

33 Keywords separated by ' - '

Ericoid mycorrhizal fungi - Metal tolerance - Yeast model system - Omics approaches

34 Foot note information

4 **Model systems to unravel the molecular mechanisms of heavy**
5 **metal tolerance in the ericoid mycorrhizal symbiosis**6 **Stefania Daghino¹ · Elena Martino¹ · Silvia Perotto¹**
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10 **Abstract** Ericoid mycorrhizal plants dominate in harsh envi-
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14 Ericaceae, metal tolerance in these plants has been mainly
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16 The mechanisms underlying plant protection by the fungal
17 symbiont are poorly understood, whereas some insights have
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19 metal tolerance in the fungal symbiont. This review will brief-
20 ly introduce the general features of heavy metal tolerance in
21 mycorrhizal fungi and will then focus on the use of “omics”
22 approaches and heterologous expression in model organisms
23 to reveal the molecular bases of fungal response to heavy
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25 *cerevisiae* has allowed the identification of several ericoid
26 mycorrhizal fungi genes (i.e., antioxidant enzymes, metal
27 transporters, and DNA damage repair proteins) that may con-
28 tribute to metal tolerance in a metal-tolerant ericoid
29 *Oidiodendron maius* isolate. Although a powerful system,
30 the use of the yeast complementation assay to study metal
31 tolerance in mycorrhizal symbioses has limitations. Thus,
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35 mycorrhization, stable genetic transformation, genomics, and
36 transcriptomic and proteomic resources.

Keywords Ericoid mycorrhizal fungi · Metal tolerance · Yeast 37
model system · Omics approaches 38

Mycorrhizal fungi protect their host plant 39
from abiotic stress, including heavy metals 40

Symbioses between plants and beneficial soil microorgan- 41
isms, such as mycorrhizal fungi, promote plant growth by 42
improving plant nutrition and competition, but they also help 43
plants to cope with several environmental stresses (Jung et al. 44
2012). For example, it has been documented by several au- 45
thors that both ectomycorrhizal (ECM) and arbuscular mycor- 46
rhizal (AM) fungi can improve drought tolerance and enhance 47
salt tolerance of their host plants (Luo et al. 2011, 2014; Ma 48
et al. 2014; Talaat and Shawky 2014). Heavy metals are an 49
important source of environmental stress because they can be 50
very toxic at above threshold concentrations. Metal-adapted 51
plant species or ecotypes survive in metal-contaminated envi- 52
ronments mainly thanks to exclusion or detoxification mech- 53
anisms (see e.g., Hall 2002; Ernst 2006; Verbruggen et al. 54
2009). However, plants can also achieve metal tolerance 55
through the association with mycorrhizal fungi. In fact, in 56
spite of some variations in metal accumulation in the host 57
plant, most studies indicate that ECM and AM plants accu- 58
mulate less metal inside their tissues and grow better than non- 59
mycorrhizal plants do when exposed to an excess of heavy 60
metals (Adriaensen et al. 2004, 2005, 2006; Audet and 61
Charest 2006; Jourand et al. 2010; Walker et al. 2004). In 62
addition to protecting the plant from excess uptake, mycorrhiz- 63
al fungi may also enhance plant internal detoxification (Luo 64
et al. 2014). 65

Plants in the family Ericaceae dominate in nutrient-poor 66
and stressful soil conditions. Metal-tolerant species and eco- 67
types have been found also in these plants and suggest specific 68

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69 adaptation mechanisms (Sharples et al. 2000a; Rossini-Oliva
70 et al. 2012). However, more important in these soil conditions
71 seems to be the association of Ericaceae with ericoid mycor-
72 rhizal (ERM) fungi, which form intracellular symbioses in
73 their fine hair roots (Fig. 1). Metal tolerance in ERM plants
74 has been linked to the stress tolerance of their fungal partners,
75 which would increase host-plant tolerance as well (Bradley
76 et al. 1981, 1982; Cairney and Meharg 2003). Soils colonized
77 by Ericaceae are generally acidic, and the low pH and anaer-
78 obic soil conditions facilitate mobilization of heavy metal ions
79 (Meharg and Cairney 2000). Bradley et al. (1981, 1982) dem-
80 onstrated for the first time the importance of ERM fungi in
81 increasing resistance of *Calluna vulgaris* to heavy metals, and
82 other authors later described metal tolerance in ERM fungal
83 isolates from sites with different pollution (Martino et al.
84 2000a; Sharples et al. 2000b; Vallino et al. 2011). Despite
85 these observations, our understanding of the mechanisms under-
86 lying plant protection by the ERM fungi is still poor,
87 whereas increasing knowledge is being gathered on the mech-
88 anisms of heavy metal tolerance in ERM fungi. In particular, a
89 number of mechanisms has been identified in metal-tolerant
90 isolates of the ERM fungus *Oidiodendron maius*, a species in
91 the Leotiomycetes (Ascomycetes) isolated from experimental
92 plots in the Niepolomice Forest (Poland), a site heavily con-
93 taminated with industrial dusts and containing high concen-
94 trations of Zn, Cd, and Al (Martino et al. 2000a, 2000b, 2002,
95 2003; Vallino et al. 2005, 2009; Abbà et al. 2011; Khouja et al.
96 2013, 2014).

97 Starting from a brief summary of the general features of
98 heavy metal tolerance in mycorrhizal fungi, this review will
99 focus on the use of yeast, a well-established fungal model
100 system, to identify genes involved in heavy metal tolerance
101 in fungi. Thanks to functional complementation of
102 *Saccharomyces cerevisiae* metal-sensitive mutants, several

103 genes that may contribute to metal tolerance were identified
104 in a heavy metal-tolerant isolate of the ERM species
105 *Oidiodendron maius*. We will also describe the features that
106 helped us to develop *O. maius* as a model system for ERM
107 fungi and some recent findings on the mechanisms of heavy
108 metal tolerance in this species.

109 Mechanisms of heavy metal detoxification 110 in mycorrhizal fungi

111 Metal elements are directly or indirectly involved in all
112 aspects of microbial growth (Gadd 1993, 2010), with several
113 of them playing essential functions (e.g., Zn, Cu, Mg, Fe)
114 and some (e.g., Cs, Al, Cd, Hg, and Pb) having no known
115 function in most organisms and being therefore already toxic
116 at low concentrations. In addition, heavy metals often influ-
117 ence the uptake and concentrations of essential elements such
118 as phosphorus and nitrogen (Krzmaric et al. 2009; Luo et al.
119 2014). Molecular recognition allows organisms to differenti-
120 ate between essential and non-essential elements and, if nec-
121 essary, to partition them in different ways. The toxicity of
122 heavy metals to both mycorrhizal fungi and their host plants
123 can result from molecular disfunctions caused by the displac-
124 ement of essential metals in biomolecules (e.g., enzymes and
125 transcription factors), from the binding of metals to thiol
126 groups, which inhibits functions of the target biomolecules,
127 and from overproduction of ROS as the consequence of
128 blocked thiol groups (Sharma and Dietz 2009; Schützendübel
129 and Polle 2002).

130 Emerging evidence suggests that the cellular mechanisms
131 involved in detoxification of excess heavy metals by mycor-
132 rhizal fungi include (similar to other fungi): (a) the biosorption
133 of metals to the fungal cell walls, (b) the binding of heavy

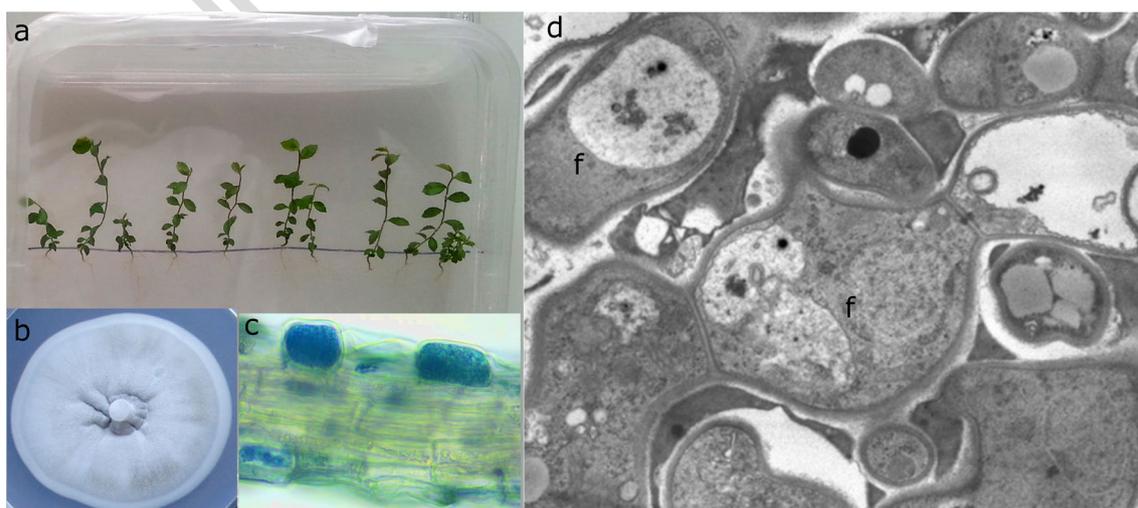


Fig. 1 General features of ericoid mycorrhiza. **a** Cocultivation of *Vaccinium myrtilloides* plantlets with *Oidiodendron maius* Zn, generating mycorrhizal plants; **b** *O. maius* Zn mycelium grown on Czapek-dox

agar medium; **c** *V. myrtilloides* root with hyphal coils of *O. maius* Zn (cotton blue staining); **d** TEM section of a mycorrhizal *V. myrtilloides* root cell; *f* indicates the fungal hyphae

134 metals to extracellular exudates and consequent possible pre-
 135 cipitation, (c) the decreased uptake and/or removal of metal
 136 ions from the cytosol via transporters located at the plasma
 137 membrane, (d) the chelation of metal ions in the cytosol by
 138 compounds such as glutathione, metallothioneins and, rarely,
 139 phytochelatins, (e) the compartmentation of metals in the vac-
 140 uole or other subcellular structures, (f) the repair of metal-
 141 damaged biomolecules, and (g) antioxidative mechanisms that
 142 allow the fungus to directly or indirectly counteract accumu-
 143 lation of ROS and oxidative stress (Bellion et al. 2006;
 144 Colpaert et al. 2011; Gallego et al. 2012; Seth et al. 2012).
 145 Some of these mechanisms are constitutively present, whereas
 146 others are only activated when metals exceed a threshold val-
 147 ue (Colpaert et al. 2011).

148 **Yeast as a model system to study fungal response**
 149 **to heavy metals**

150 Model systems are important tools to unravel the molecular
 151 mechanisms underlying biological processes. *S. cerevisiae* in
 152 particular is an attractive model organism due to the fact that it
 153 is very easy to maintain in the lab and has a fast life cycle. In
 154 addition, its genome has been fully sequenced (Goffeau et al.
 155 1996), thus making genetic manipulation easier and analyses
 156 based on high throughput approaches (i.e., “omics” ap-
 157 proaches such as genomics, transcriptomics, proteomics,
 158 metabolomics, and phenomics) more informative than in other
 159 organisms.

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

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

181 The screening of the yeast deletion mutant collection to
 182 assess the role of non-essential genes in the response to heavy
 183 metals (Zn, Cd, Hg, Cu, Ag, Cr, As and Ni) revealed a major

184 role of the vacuole for metal sequestration and detoxification. 184
 185 A wide range of additional cellular functions likely involved in 185
 186 general stress response and repair of damage caused by metals 186
 187 were also identified, such as the GSH and reduced sulfur me- 187
 188 tabolism, metal chelation, antioxidant defense, protein turn- 188
 189 over, mRNA decay and trafficking, structural and functional 189
 190 integrity of the membranes, and DNA repair. The chemical 190
 191 properties of the metals likely define the responsive genes and 191
 192 the cell toxicity effects. For example, it is not surprising that 192
 193 Cd and Hg raised similar responses because they share a simi- 193
 194 lar thiophilicity and lack of redox activity, as well as Mn, Ni, 194
 195 Zn, and Co, that are all non-redox transition metals, whereas 195
 196 Fe(III) is redox-active and was the most divergent metal in- 196
 197 vestigated (Jin et al. 2008; Ruotolo et al. 2008). In addition, 197
 198 Ruotolo et al. (2008) suggested that components of the high- 198
 199 affinity Fe transport pathway contributed to the yeast tolerance 199
 200 to Cu, Mn, Ni, Co, and Zn, but not to Fe, suggesting that Fe 200
 201 homeostasis requires different mechanisms. Bleackley et al. 201
 202 (2011) suggested a lack of metal-specificity based on the re- 202
 203 sults of a new deletome screening showing that Mn, Ni, Zn, 203
 204 and Co sensitivity was common to a number of deletion 204
 205 strains. These authors discussed that promiscuity in metal 205
 206 binding in proteins likely preceded metal binding specificity 206
 207 during evolution, and the overlap in tolerance pathways may 207
 208 be interpreted as a relic of metal binding promiscuity. 208
 209 However, most of the vacuolar deletion strains were sensitive 209
 210 to Mn, Zn, Ni, and Co (all of which are stored in the vacuole), 210
 211 but not to Fe. 211

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

214 Omics approaches in yeast have been instrumental to investi- 214
 215 gate fungal responses to heavy metals, and they have 215
 216 unraveled common as well as metal-specific pathways. 216
 217 However, *S. cerevisiae* is not very tolerant to heavy metals, 217
 218 and whereas its deletome/transcriptome can help to explain 218
 219 stress response of fungi when exposed to heavy metals, it 219
 220 has limitations to unravel mechanisms underlying heavy metal 220
 221 tolerance in metal-tolerant filamentous fungi. Other method- 221
 222 ologies, employing the yeast model system for heterologous 222
 223 expression and functional complementation of deletion mu- 223
 224 tants, have been helpful in identifying genes from metal- 224
 225 tolerant ECM and AM mycorrhizal fungi (Courbot et al. 225
 226 2004; González-Guerrero et al. 2005; Lanfranco et al. 2002). 226
 227 Functional complementation of yeast mutations was used for 227
 228 the first time in ERM fungi as a targeted approach to demon- 228
 229 strate the role of Cu/Zn superoxide dismutase (SOD) enzyme 229
 230 in metal tolerance (Vallino et al. 2009). The synthesis of anti- 230
 231 oxidant enzymes such as catalases, peroxidases, and SODs is 231
 232 known to protect fungi from the oxidative stress caused by 232
 233 heavy metals (Guelfi et al. 2003; Jacob et al. 2001; Todorova 233

234 et al. 2008). In particular, SODs play a protective role against
 235 free superoxide radical toxicity (Fridovich 1995) and their
 236 induction by heavy metals has been described in plants, ani-
 237 mals, and microorganisms (Chongpraditnun et al. 1992; Yoo
 238 et al. 1999; Vido et al. 2001). Exposure of *O. maius* Zn, a
 239 metal-tolerant isolate derived from the Niepolomice (Poland)
 240 contaminated soil, to Zn and Cd increased the amount and
 241 activity of both intracellular and extracellular SOD enzymes
 242 that could help both the ERM fungus and the host plant to
 243 cope with ROS formation (Chiapello et al. 2015; Martino et al.
 244 2002; Vallino et al. 2009). As these enzymes are
 245 metalloenzymes, like most oxidoreductases, the increased
 246 production of an extracellular Cu/Zn SOD in *O. maius* Zn
 247 may also reduce metal toxicity thanks to its metal binding
 248 capacity (Vallino et al. 2009). A metal-sensitive yeast mutant
 249 lacking this enzyme regained metal tolerance to Zn, Cu, and
 250 Cd when transformed with the *O. maius* Zn full-length cDNA
 251 coding for this enzyme.

252 Targeted approaches, such as yeast functional complemen-
 253 tation with the *OmSod1* gene, normally rely on existing
 254 knowledge and may be helpful confirming the role of individ-
 255 ual components in heavy metal tolerance, but they would miss
 256 so far unidentified mechanisms that can be better addressed by
 257 untargeted approaches. Untargeted approaches are in fact an
 258 important source of novel information, especially if they are
 259 supported by functional assays (Ruytinx et al. 2011). Metal-
 260 sensitive yeast mutants have been used to screen by functional
 261 complementation whole cDNA libraries from mycorrhizal
 262 fungi (Leonhardt et al. 2014a; Osobová et al. 2011; Ramesh
 263 et al. 2009) in order to identify genes capable of conferring to
 264 the transformants the ability to grow in metal-containing me-
 265 dia. The same approach has lead, in the metal-tolerant ERM
 266 fungus *O. maius* Zn, to the identification of some metal trans-
 267 porters (Khouja et al. 2013), but also to the discovery of the
 268 novel protein OmFCR, a member of the PLAC8-domain con-
 269 taining proteins, likely involved in DNA damage repair (Abbà
 270 et al. 2011). These genes are described in the following
 271 paragraphs.

272 ***O. maius* Zn gene coding for metal transporters**

273 Membrane transporters can reduce heavy metal toxicity be-
 274 cause they can regulate cytoplasmic metal concentrations ei-
 275 ther by limiting metal uptake or by increasing metal efflux
 276 and/or compartmentation in cell organelles (Pócsi 2011). In
 277 order to identify membrane transporters involved in heavy
 278 metal tolerance in *O. maius* Zn, functional screening of a
 279 cDNA library obtained from this ERM fungus growing on
 280 Cd-amended medium was performed in the zinc-sensitive
 281 $\Delta zrc1$ mutant of *S. cerevisiae*. Two full-length cDNAs were
 282 isolated and further characterized in yeast, respectively
 283 encoding OmZnT1, a member of the cation diffusion

284 facilitator family of zinc transporters, and OmFET, a member
 285 of the iron permease family (Khouja et al. 2013; Fig. 2b).

286 Zn homeostasis has been largely investigated in yeast,
 287 whereas much less is known in filamentous fungi. In mycor-
 288 rhizal fungi, although many putative Zn-transporter genes
 289 have been identified *in silico* (e.g., Tamayo et al. 2014), only
 290 a few have been functionally characterized in yeast: the
 291 *RaZIP1* and *RaZIP2* from *Russula atropurpurea* (Leonhardt
 292 et al., 2014b), *HcZnt1* from *Hebeloma cylindrosporum*
 293 (Blaudez and Chalot 2011), *GiZnT1* from *Rizophagus*
 294 *irregularis* (renamed *RiZnT1*; González-Guerrero et al.
 295 2005), and *OmZnT1* and *OmFET* from *O. maius* Zn (Khouja
 296 et al. 2013). In yeast, OmZnT1 was located in the ER mem-
 297 brane and was able to restore growth of Zn and Co sensitive
 298 mutants lacking vacuolar transporters, suggesting that it could
 299 detoxify zinc by delivery and compartmentation into the ER,
 300 a common strategy of metal tolerance. Similarly, the ER-
 301 resident CDF proteins ZHF1 from the yeast
 302 *Schizosaccharomyces pombe* (Clemens et al. 2002) and
 303 *HcZnT1* from the basidiomycete *Hebeloma cylindrosporum*
 304 (Blaudez and Chalot 2011) have been demonstrated to confer
 305 zinc tolerance in yeast. The release of the *O. maius* Zn genome
 306 sequence (<http://genome.jgi.doe.gov/>; Kohler et al. 2015)
 307 revealed the presence of two other putative Zn-CDF trans-
 308 porters in this organism.

309 OmFET is a low-affinity iron transporter that has also been
 310 found in other filamentous fungi, but it has been fully charac-
 311 terized only in *S. cerevisiae* (Kosman 2003), showing relative-
 312 ly low substrate specificity. Measurement of intracellular ion
 313 concentration indicates that yeast transformants constitutively
 314 expressing OmFET contained significantly less Zn than cells
 315 did harboring the empty vector, which would explain the posi-
 316 tive selection of *OmFET* during the screening of the *O. maius*
 317 cDNA library (Khouja et al. 2013). Although this transporter
 318 belongs to the iron permeases family, we could not find sig-
 319 nificant iron accumulation in the OmFET-expressing yeast, as
 320 compared to control cells, while the magnesium content was
 321 always significantly higher in OmFET-expressing cells than in
 322 control cells. It was thus suggested that OmFET could en-
 323 hance zinc tolerance in yeast by increasing the cellular content
 324 of magnesium, which has a general protective effect against
 325 different heavy metal cations such as manganese, copper,
 326 nickel, cadmium, and cobalt in yeast (Blackwell et al. 1998;
 327 Joho et al. 1991; Karamushka and Gadd 1994) and zinc and
 328 cadmium in plants (Kupper and Kochian, 2010; Pedler et al.
 329 2004).

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

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

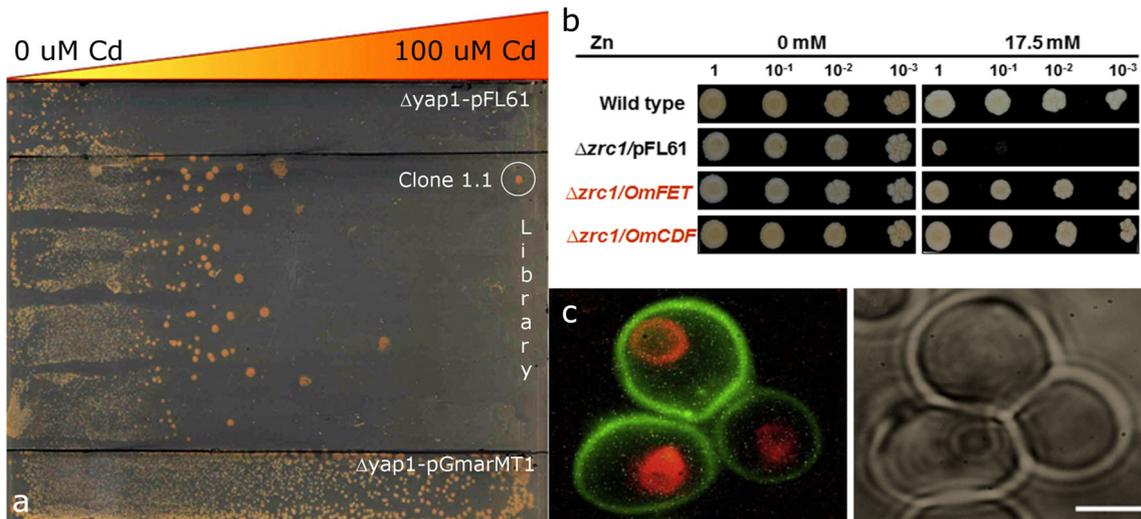


Fig 2 Heterologous expression in yeast of *O. maius* Zn genes. **a** The *yap1*-deficient yeast strain was transformed with a pFL61-cDNA library of *Oidiodendron maius* Zn exposed to CdSO₄ and, the yeast cells were spread on SD-agar plates containing a linear concentration gradient (0–100 mM) of CdSO₄: the clones growing at the highest concentrations

were all expressing *OmFCR1*; **b** functional complementation of the metal-sensitive $\Delta zrc1$ yeast strains by *OmFET* and *OmZnT1*; **c** localization of *OmFET*/GFP on the yeast plasma membrane by epifluorescence microscopy, and corresponding bright field image

335 (*Δyap1*). The new gene was called *O. maius* fungal cadmium
 336 resistance 1 (*OmFCR1*) because of the structural and function-
 337 al similarities with its ortholog in *Arabidopsis thaliana* plant
 338 cadmium resistance (*AtPCR*). These genes both harbor a
 339 PLAC-8 (or DUF614) conserved domain whose function re-
 340 mains unknown despite a number of studies that attributed
 341 different roles to members of this protein family, ranging from
 342 the control of cell cycle and cell size in both animal and plants
 343 (Frery et al. 2000; Guo et al. 2010; Jimenez-preitner et al.
 344 2011, 2012; Rogulski et al. 2005) to a function in cadmium
 345 resistance for *AtPCR* (Song et al. 2004). *OmFCR1* is likely to
 346 confer Cd resistance by interacting with components of the
 347 mismatch repair (MMR) system involved in DNA damage
 348 repair (Abbà et al. 2011; Fig. 2a). More recently, another gene
 349 which also harbors a PLAC-8 domain was identified in the
 350 genome of *O. maius* Zn. This gene, called *OmFCR2*, was able
 351 to rescue the Cd-sensitive phenotype in mutant yeast, although
 352 less pronounced than *OmFCR1* (Di Vietro et al. 2014).
 353 Expression of *OmFCR1* in *O. maius* Zn, as measured by
 354 real-time qPCR, significantly increased after 24 h of Cd expo-
 355 sure, while the expression of *OmFCR2* was constant and gen-
 356 erally lower than *OmFCR1* expression. Hence, these two
 357 genes share a similar function in Cd response but show a
 358 different expression trend, thus suggesting a possible modula-
 359 tion of the response to Cd, just like it would be expected for
 360 paralogs (Gabaldon and Koonin 2013). Besides, both
 361 *OmFCR1* and *OmFCR2* promoter regions harbor putative
 362 metal response elements (MRE), suggesting that the metal-
 363 mediated induction has been conserved after duplication (Di
 364 Vietro et al. 2014). The generation of *OmFCR1* knock-out
 365 mutants in *O. maius* Zn had not resulted in a Cd-sensitive
 366 phenotype, and a possible explanation is that *OmFCR2* could

contribute to the resistant phenotype in the *OmFCR1* knock- 367
 out mutants, together with a number of cellular/molecular re- 368
 sponses activated by the fungus and described in other studies 369
 (Martino et al. 2000b, 2003; Khouja et al. 2013; Vallino et al. 370
 2009). 371

***O. maius* as a model system for ericoid mycorrhizal fungi** 372

Yeast has been very helpful in the identification of heterolo- 373
 gous genes involved in metal tolerance from mycorrhizal fun- 374
 gi, by both targeted and untargeted functional complementa- 375
 tion. However, the use of the yeast system has limitations for 376
 the study of metal tolerance mechanisms in mycorrhizal fungi 377
 because it mainly reveals mechanisms based on individual 378
 molecular components (e.g., metal transporters, antioxidant 379
 enzymes, etc.) rather than more complex cellular functions. 380
 In addition, some of the mechanisms that operate in mycor- 381
 rhizal fungi may also confer protection to the host plant, a 382
 potential feature that could not be tested in yeast. Hence, the 383
 elucidation of the mechanisms of heavy metal homeostasis in 384
 mycorrhizal fungi and their possible roles in plant protection 385
 require the development of mycorrhizal model systems, possi- 386
 bly with characteristics of heavy metal tolerance, ease of 387
 laboratory handling, knowledge of the genome sequence, 388
 and availability of genetic transformation protocols. For 389
 ERM, *O. maius* Zn is emerging as a model system to investi- 390
 gate cellular processes related to heavy metal tolerance. This 391
 ascomycete can be easily grown in vitro, where it produces 392
 asexual conidia containing a single haploid nucleus, which 393
 can germinate to produce a homokaryotic mycelium. In addi- 394
 tion to the haploid genome and easy culturing, tools have been 395
 developed for *O. maius* over the years, such as genetic 396

397 transformation and omics databases. This ERM fungus is also
398 a relatively easy system to study the expression and function
399 of fungal genes during mycorrhizal interactions (Kohler et al.
400 2015), but it will be also an interesting model system for the
401 functional study of genes from other less genetically tractable
402 mycorrhizal fungi by heterologous expression, and for the
403 identification of common pathways in mycorrhizal interac-
404 tions. For example, constitutive expression in *O. maius* Zn
405 of an AM fungal gene induced during arbuscule development
406 resulted in a higher percentage of *Vaccinium myrtillus* root
407 colonization (Lanfranco et al. unpublished data).

408 **Omics approaches to identify mechanisms of heavy** 409 **metal tolerance in *O. maius* Zn**

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

428 Comparative high-throughput proteomics, another “omic”
429 approach, was more recently applied to investigate protein
430 accumulation in *O. maius* Zn exposed to zinc and cadmium
431 (Chiapello et al. 2015). Cadmium selectively induced molec-
432 ular chaperones of the Hsp90 family, cytoskeletal proteins,
433 and components of the translation machinery, while zinc sig-
434 nificantly upregulated metabolic pathways related to energy
435 production and carbohydrate metabolism, suggesting that ad-
436 aptation of this isolate to Zn exposure mainly involved the
437 primary metabolism. Common proteins induced by the two
438 metal ions were the antioxidant enzyme Cu/Zn SOD (further
439 supporting earlier experiments by Abbà et al. 2009 and
440 Vallino et al. 2009) and ubiquitin. The covalent attachment
441 of ubiquitin to lysine residues of proteins is a post-
442 translational modification originally described as a destruction
443 tag that directs misfolded or disused proteins to the protea-
444 some (Hall 2002). Some components of the proteasome were
445 identified by 2-DE in the mycelium exposed to both cadmium
446 and zinc suggesting the induction of the proteolytic activity

eliminating ubiquitinated proteins as defence mechanism. 447
Several proteins involved in ubiquitin-dependent proteolysis 448
were also identified by Muller et al. (2007) when comparing 449
the gene expression profiles of a Zn-tolerant and a Zn- 450
sensitive *Suillus luteus* isolate exposed to increasing external 451
zinc concentrations. 452

453 An interesting protein identified by shotgun proteomics 453
and induced in *O. maius* Zn by both cadmium and zinc was 454
the enzyme agmatinase, a key enzyme in the biosynthesis of 455
polyamines (Dudkowska et al. 2003). Identification of 456
agmatinase in the *O. maius* proteome in response to metals 457
is very intriguing. Polyamines are positively charged small 458
molecules found in prokaryotic and eukaryotic cells; putres- 459
cine and spermidine, in particular, are believed to occur in all 460
living cells and to be implicated in many fundamental cellular 461
processes (Igarashi and Kashiwagi 2000; Kusano et al. 2008). 462
In plants, polyamine accumulation appears to be a universal 463
response to stress, including toxic heavy metal concentrations 464
(Alcázar et al. 2010; Minocha et al. 2014). In fungi, poly- 465
amines are essential to support growth and to regulate a wide 466
variety of biological processes (Davis 1996; Valdés-Santiago 467
et al. 2010), but little is known about their possible role in 468
stress tolerance (Valdés-Santiago and Ruiz-Herrera 2014). 469
Polyamine accumulation in response to heavy metals was in- 470
vestigated in the ECM fungus *Paxillus involutus*, where lead 471
and zinc exposure specifically increased cellular concentra- 472
tions of some polyamines (Zarb and Walters 1995; 1996). 473
Although the role of polyamines in the ERM fungal response 474
to environmental stress requires further investigations, the re- 475
sults by Chiapello et al. (2015) suggest that the biosynthetic 476
pathway leading to their formation is induced by both zinc and 477
cadmium in *O. maius* Zn. 478

479 **Genetic transformation of *O. maius* Zn to identify** 480 **mechanisms of metal tolerance**

481 Further tools have been developed for *O. maius* Zn and in- 481
clude protocols for stable genetic transformation (Fig. 3) by 482
both PEG- and *Agrobacterium*-mediated transformation 483
(Martino et al. 2007; Abbà et al. 2009). Although several 484
ECM fungi have been stably transformed (Combiér et al. 485
2003; Kemppainen et al. 2005; Marmeisse et al. 1992; Pardo 486
et al. 2002; Rodríguez-Tovar et al. 2005), *O. maius* Zn re- 487
mains so far the only example of genetic transformation of 488
an endomycorrhizal fungus. Genetic transformation of 489
O. maius Zn has been used to disrupt gene functions in order 490
to identify possible alterations in the fungal phenotype. The 491
complete and stable inactivation of a target gene via homolo- 492
gous recombination at the wild-type locus (gene knock-out) is 493
in fact the most direct way to explore gene function, and it was 494
successfully applied in *O. maius* Zn for the first time among 495
mycorrhizal fungi. The SOD1-null mutant showed an 496

Mycorrhiza

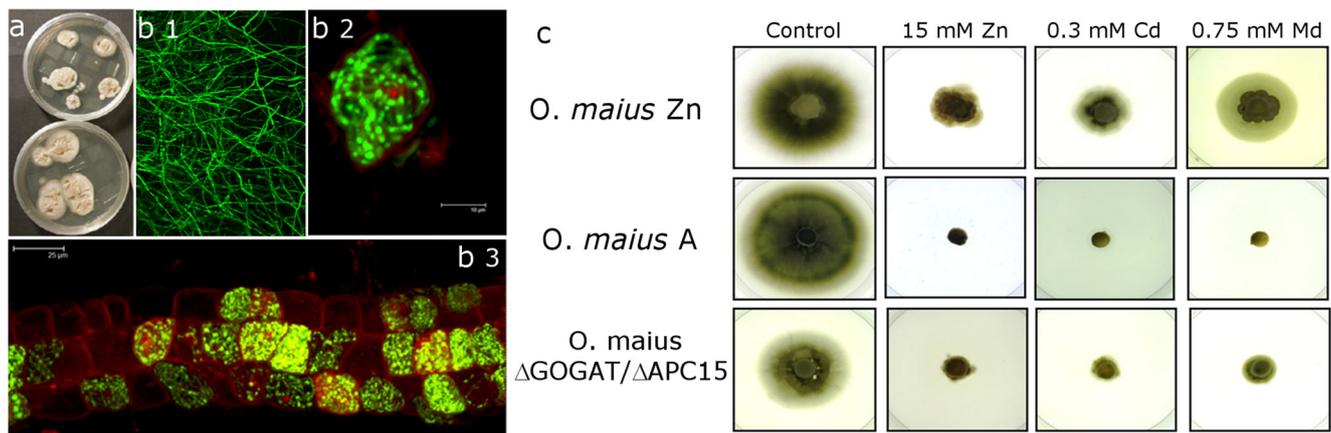


Fig 3 Genetic manipulation of *Oidiodendron maius* Zn. **a** *O. maius* Zn mutants selected for their hygromycin B resistance after *Agrobacterium tumefaciens*-mediated transformation; **b**. confocal microscopy images of *O. maius* Zn expressing EGFP (**b.1** free living mycelium; **b.2** hyphal coil; **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 ZnSO₄, 0.3 mM CdSO₄, or 0.75 mM menadione

497 imbalanced ROS homeostasis as well as a decreased Cd and
 498 Zn tolerance and a decrease of the formation of mycorrhizal
 Q1 499 coils with respect to the wild-type (WT) strain. These results
 500 suggested that the ROS scavenging has an important role not
 501 only in the stress defence but also in the signaling between
 502 *O. maius* Zn and its host plant (Abbà et al. 2009).

503 In addition to the disruption of target genes by homologous
 504 recombination, genetic transformation can be used for random
 505 insertional mutagenesis. This approach has been used in
 506 *O. maius* Zn to build up a library of more than 2000 random
 507 mutants. This library was screened for sensitivity to heavy
 508 metals (Zn and Cd) and oxidative stress (menadione), and a
 509 number of mutants with altered phenotype and/or impaired
 510 growth in one or more of these conditions were selected.
 511 One of these mutants, in addition to an altered metal stress
 512 tolerance (Figs. 3c and 4), also showed impaired N-
 513 metabolism and was further characterized (Khouja et al.
 514 2014). The glutamate synthase (GOGAT), a key enzyme in
 515 nitrogen metabolism, and its adjacent gene, *APC15*, were par-
 516 tially deleted. Genetic transformation was used to
 517 recomplement the disrupted *OmAPC15* gene with the func-
 518 tional *O. maius* Zn gene, and assays on two *OmAPC15*-
 519 recomplemented strains ascribed the metal sensitive pheno-
 520 type to the deletion of the *OmGOGAT* gene. The
 521 *OmGOGAT*-deleted strain also showed a reduction of the glu-
 522 tamine synthetase (GS) activity and an upregulation of the
 523 alternative NADP-glutamate dehydrogenase pathway for glu-
 524 tamate biosynthesis, suggesting a strong alteration of the N-
 525 assimilation pathway. Unless they were supplemented with
 526 glutamine, *O. maius* Zn transformants lacking OmGOGAT
 527 were very sensitive to zinc. A number of studies in plants
 528 demonstrate the significance of nitrogen containing metabo-
 529 lites in the response to heavy metals (Sharma and Dietz 2006)
 530 and report in particular the involvement of glutamine and GS

enzyme activity in the tolerance mechanisms to oxidative 531
 stress induced by metals (Hradilová et al. 2010; Ker and 532
 Charest, 2010; Kieffer et al. 2008; Wang et al. 2008). 533
 Glutamine synthetase was found to be upregulated by Cd in 534
 several plant species (Kieffer et al. 2008; Rana et al. 2008; 535
 Sarry et al. 2006; Wang et al. 2008) and was positively corre- 536
 lated with Cd tolerance in the hyperaccumulator *Noccaea* 537
caerulescens (Tuomainen et al. 2006). Nitrogen uptake via 538
 glutamine/glutamate cycle is also linked to the glutathione 539
 biosynthesis (Li et al. 1993; Matés et al. 2002), and a possible 540
 role of glutathione is to reduce the concentration of free metal 541
 ions in the cell and prevent an increase in the production of 542
 reactive oxygen species under heavy metal stress (Xu et al. 543
 2009). This could suggest that a possible reduced glutathione 544
 biosynthesis in the *OmGOGAT*-deleted strain would 545

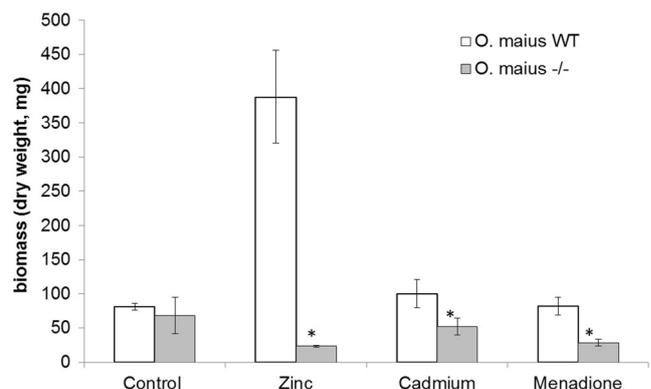


Fig. 4 Stress tolerance of the *Oidiodendron maius* Zn wild-type (white bars, *O. maius* WT) and the *O. maius* Δ GOGAT/ Δ APC15 strain (light gray bars, *O. maius* -/-). The two fungal strains were grown in liquid media supplemented or not (control) with 10 mM ZnSO₄, 0.1 mM CdSO₄ or 0.5 mM of menadione.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

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

556 Pythochelatins play an important role in metal tolerance in
557 plants, and gene coding for pythochelatin synthase, or putative
558 homologs of this enzyme, have been recently found in some
03/Q2 559 fungal genomes (Bolchi 2011, Shine 2015). Phytochelatin
560 synthase genes seem to be absent in *O. maius* Zn (unpublished
561 data), making this fungus an interesting system for heterolo-
562 gous expression of this gene, in order to evaluate whether it
563 confers higher metal tolerance and/or whether it affects the
564 expression of other defense genes.

565 Perspectives for the study of metal tolerance in ERM 566 fungi

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

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

592 Some indications of adaptive metal tolerance were also
593 reported for *O. maius* by Vallino et al. (2011), who investigat-
594 ed a number of fungal isolates derived from soils with differ-
595 ent pollutants, namely a serpentine site enriched in Cr and Ni,

an industrial soil mainly contaminated with Cd and Zn, and a 596
non-polluted soil. These *O. maius* isolates showed a statisti- 597
cally significant difference in their ability to grow in the pres- 598
ence of the metal contaminants typical of the site of origin. 599
The isolates more tolerant to Cr and Ni were those originated 600
from the serpentine site, while the isolates more tolerant to Zn 601
and Cd were those from the industrially polluted site enriched 602
in these contaminants (Vallino et al. 2011). Some genetic fea- 603
tures were further investigated in these isolates. In particular, 604
DNA mutation rate (in terms of base substitution and inser- 605
tion/deletions) was assessed for specific regions of the fungal 606
genome that have different significance in metal tolerance: the 607
“functional” gene coding for the Cu/Zn SOD, already demon- 608
strated to play a role in metal tolerance (Vallino et al. 2009), 609
and the “neutral” ribosomal ITS gene. *O. maius* isolates from 610
all sites, polluted and non-polluted, showed higher mutation 611
rates in the functional *Sod1* locus, important for fungal surviv- 612
al, than in the neutral ITS locus (Vallino et al. 2011). In addi- 613
tion, *O. maius* isolates from heavily polluted industrial soils 614
showed a significantly higher mutation rates in the *Sod1* locus 615
than fungi from less polluted or non-polluted sites. The accu- 616
mulation of mutations was not the result of a random process 617
because a higher mutation rate was calculated for the *Sod1* 618
promoters of metal-exposed than non-exposed isolates, while 619
the mutation rate was similar when the coding sequence was 620
considered (Vallino et al. 2011). Although limited to a single 621
locus and to a small population of ERM fungi, these observa- 622
tions would indicate that mutagenesis induced by environ- 623
mental stress may target specific gene regions and suggest a 624
rapid evolution of key pathways, like stress signaling, driven 625
by the need of defense of the organism (Nikolaou et al. 2009). 626

627 It will be therefore interesting to extend these types of anal-
628 yses to whole genomes. Sequencing of fungal genomes is
629 becoming relatively simple and cost-effective and, based on
630 the already fully sequenced genome of *O. maius* Zn,
631 resequencing of several other *O. maius* isolates from metal-
632 tolerant and metal-sensitive populations should provide addi-
633 tional information on the evolution of metal tolerance molec-
634 ular mechanisms.

635 Conclusions

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

- 646 databases, as well as genetic tools, have been developed for
647 the metal-tolerant isolate *O. maius* Zn, and they have been
648 instrumental in identifying not only tolerance mechanisms
649 already known from other organisms but also novel molecular
650 components and metabolic pathways involved in metal toler-
651 ance. Multiple mechanisms likely enable metal-tolerant ERM
652 fungi to protect themselves and their host plant from toxic
653 compounds. Further use of omics approaches is already in
654 progress to compare metal-tolerant and metal-sensitive ERM
655 isolates, as well as transcriptomic analyses of the host plant
656 under different conditions. These data will help not only in
657 dissecting the molecular and cellular pathways involved in
658 heavy metal tolerance in ERM fungi, but hopefully also in
659 revealing the mechanisms underlying mycorrhiza-modulated
660 host plant tolerance to heavy metals.
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AUTHOR PLEASE ANSWER ALL QUERIES.

- Q1. “wild-type” has been provided as definition for TW. Please check if correct and amend as necessary.
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