Gene expression and role in cadmium tolerance of two PLAC8 containing proteins identified in the ericoid mycorrhizal fungus Oidiodendron maius

Gene expression and role in cadmium tolerance of two PLAC8-containing proteins identified in the ericoid mycorrhizal fungus *Oidiodendron maius*

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Highlights

•A new PLAC8-protein, OmFCR2, was found in the *Oidiodendron maius* genome.
•Both OmFCR1 and OmFCR2 proteins confer cadmium-resistance when expressed in yeast.
•OmFCR1 and OmFCR2 promoters contain a metal-responsive element.
•*OmFCR1* expression is induced in *O. maius* exposed to Cd, while *OmFCR2* is stable.
•*OmFCR1* gene knock-out does not specifically affect *O. maius* phenotype on Cd.

Abstract

Fungi living in heavy metal polluted soils have evolved different cellular and molecular systems to adapt and survive in these harsh environments. *Oidiodendron maius* Zn is an ericoid mycorrhizal fungus previously shown to be highly tolerant to zinc thanks to antioxidative enzymes and membrane transporters. A novel gene, *OmFCR1*, was recently identified from this fungus because it conferred strong cadmium tolerance when expressed in yeast. *OmFCR1* codes for a protein belonging to the PLAC8 family and physically interacts in yeast with the mismatch repair system, involved in DNA damage repair. The *O. maius* Zn genome also contains another gene – named *OmFCR2* – that codes for a protein sharing with OmFCR1, the PLAC8 domain and other sequence similarities.

In this work, we analyzed gene expression of *OmFCR1* and *OmFCR2* in the fungus *O. maius* Zn when exposed to cadmium, the ability of *OmFCR2* to confer cadmium tolerance when expressed in yeast, and the growth of OmFCR1-null mutants of *O. maius* Zn upon cadmium exposure. Although *OmFCR2* was also able to confer some cadmium tolerance to yeast, the different expression pattern of these two genes would suggest different roles in *O. maius* Zn.

Keywords

- Ericoid mycorrhizae;
- Genetic transformation;
- Heavy metals;
- PLAC8 domain;
- Stress response

Introduction

Environmental pollution caused by heavy metals is increasing, especially in soil, as a result of both anthropogenic and natural activities (Khan *et al.* 2010). In particular, non essential heavy metals – *i.e.* those playing no known physiological function in living organisms and represented mainly by cadmium, lead, arsenic and mercury – could be very toxic even at low concentrations (Ali *et al.* 2013). Molecular mechanisms that allow plants to cope with heavy metal stress are, even if not completely understood, intensively studied (Lin & Aarts 2012), and there are practical applications of some metal resistant plants in soil bioremediation (Miransari 2011). Rhizospheric microorganisms such as bacteria and fungi can intensify metal toxicity by increasing metal bioavailability to plants or, on the contrary, they can protect plants by chelating or precipitating free metals in soil (Leyval et al., 1997, Schützendübel and Polle, 2002 and Gadd, 2010). Mycorrhizal fungi are important components of the rhizosphere of most terrestrial plants, and an influence on metal uptake and tolerance in the host plant has been envisaged because of their pivotal role in plant mineral nutrition (Gamalero et al., 2009 and Ma et al., 2013). Beside the extensively studied arbuscular and ectomycorrhizal fungi, ericoid mycorrhizal fungi also have an established role in increasing plant metal tolerance (Perotto *et al.* 2012). These fungi form endomycorrhizal symbioses with plants in the *Ericales*, often capable of colonizing acidic soils poor in essential minerals but high in toxic non essential metals. In these soils, mycorrhizal fungi that confer metal tolerance are crucial to increase plant fitness.

Oidiodendron maius Zn is an ericoid mycorrhizal fungus isolated from *Vaccinium myrtillus* roots collected in a patch of soil contaminated with heavy metals in the Niepolomice forest in Poland (Martino *et al.* 2000). When compared with conspecific isolates from non polluted soil, *O. maius* Zn showed high tolerance to zinc and cadmium (Lacourt et al., 2000, Martino et al., 2000 and Martino et al., 2003). Subsequent studies have tried to identify the molecular components responsible for the high metal tolerance observed in this isolate (Martino *et al.* 2002). For example, a targeted approach has demonstrated the role of antioxidant enzymes in zinc resistance (Abbà et al., 2009, Vallino et al., 2009 and Khouja et al., 2013).

Functional complementation in *Saccharomyces cerevisiae* is a non targeted approach widely used to identify genes involved in specific cellular processes, including metal tolerance (Kampfenkel *et al.* 1995). This method has been recently used to better understand the mechanisms involved in metal tolerance in *O. maius* Zn. The functional screening of a metal-sensitive strain of *S. cerevisiae* transformed with a cDNA library of *O. maius* Zn mycelia grown on zinc-amended medium has led, for example, to the identification of two metal transporters capable of conferring metal tolerance to yeast (Khouja *et al.* 2013).

Another experiment of yeast functional complementation used a cDNA library constructed from *O. maius* Zn grown in cadmium-amended medium and led to the isolation of a novel gene that conferred strong cadmium resistance to yeast (Abbà *et al.* 2011). This gene coded for a small (179 amino acids) protein containing only one conserved domain, the PLAC8 or DUF614 domain, which can be found in many proteins of fungi, plants and animals. This gene was named *Oidiodendron maius* Fungal Cadmium Resistance (*OmFCR*) because of its sequence similarity with the *Arabidopsis thaliana* Plant Cadmium Resistance (*AtPCR*) gene (Song *et al.* 2004).

In the past decade, some PLAC8-containing proteins have been studied in plants and animals, but, despite extensive work, no clear function could be attributed to this domain (Song *et al.* 2011). Only AtPCR and similar proteins in rice and other organisms (Song *et al.* 2004) seem to confer resistance to cadmium, whereas other PLAC8-containing proteins from plants seem to regulate cell size and number (Frary et al., 2000 and Guo et al., 2010). Animal PLAC8-containing proteins play various

roles in animals, from control of cell growth (Rogulski *et al.* 2005), to host defense against pathogens (Ledford et al., 2012 and Ledford et al., 2007), or brown and white fat development and regulation (Jimenez-preitner et al., 2011 and Jimenez-preitner et al., 2012).

O. maius FCR (hereafter called OmFCR1) is the only PLAC8-containing protein from fungi characterized so far (Abbà *et al.* 2011). When expressed in yeast, OmFCR1 conferred strong cadmium resistance through mechanisms that did not include the two major detoxification systems involved in yeast response to Cd stress, namely the YCF1 transporter and the YAP1-dependent pathway. The protein Ycf1 is an ABC transporter, which limits Cd toxicity by translocating GSH-conjugated Cd into the vacuole (Li *et al.* 1996). YAP1 is a zinc-finger transcription factor regulating the expression of genes involved in the oxidant-stress response (Gomes *et al.* 2005).

The nuclear localization observed for OmFCR1 in yeast, its genetic interaction with the yeast DNA damage repair kinase DUN1 and the physical interaction with the mismatch repair protein MLH3 suggest that the mechanism of cadmium tolerance may involve DNA repair (Abbà *et al.* 2011). This hypothesis is intriguing because cadmium, unlike many other genotoxic metals, does not damage DNA directly through the generation of reactive oxygen species (ROS), but it targets proteins that are directly or indirectly involved in DNA repair and in antioxidant defense, thus altering their functions and ultimately inflicting toxic, mutagenic and carcinogenic effects (Clark and Kunkel, 2004, Banerjee and Flores-Rozas, 2005, Kunkel and Erie, 2005 and Wieland et al., 2009).

As the complete genome sequence of *O. maius* Zn became available (http://genome.jgi.doe.gov/programs/fungi/index.jsf) we searched it for genes coding for proteins similar to OmFCR1. Another gene featuring the PLAC8 domain, called *OmFCR2*, was identified and found to confer cadmium tolerance in yeast, although to a lesser extent when compared to *OmFCR1*. The heterologous yeast model system was instrumental to reveal the possible functions of the OmFCR proteins, but a complete picture of the pathways involving these proteins is hampered by the fact that no homologues of these genes could be found in the *S. cerevisiae* genome. To better understand their role in cadmium tolerance in the ericoid mycorrhizal fungus *O. maius* Zn, a far less tractable organism than yeast, we have investigated their expression profiles upon cadmium exposure, and studied the phenotype of OmFCR1-null mutants grown in the absence and presence of cadmium.

Materials and methods

Oidiodendron maius strains and growth conditions

All *O. maius* strains used in this work are summarized in Table 1. Wild-type *O. maius* Zn was previously described by Martino *et al.* (2000) and deposited as strain CLM1381 in the Mycotheca Universitatis Taurinensis (MUT), a fungal collection at the Department of Life Sciences and Systems Biology, University of Turin (Italy). The mutant and the yeast strains have been deposited in the same collection.

Table 1.

O. maius Zn and yeast strains used in the experiments.

Strains	Plasmid	Reference
Oidiodendron maius Zn – WT	/	CLM1381 (Martino *et al.* 2000)
Oidiodendron maius Zn – Random mutant-a	pCAMBIA0380-Hyg_cassette	MUT 63
Oidiodendron maius Zn – Random mutant-b	pCAMBIA0380-Hyg_cassette	MUT 70
Oidiodendron maius Zn – Random mutant-c	pCAMBIA0380-Hyg_cassette	MUT 71
Oidiodendron maius Zn – ΔFCR1-a	pCAMBIA0380-Hyg_cassette-OmFCR_del	MUT 65
Oidiodendron maius Zn – ΔFCR1-b	pCAMBIA0380-Hyg_cassette-OmFCR_del	MUT 67
Oidiodendron maius Zn – ΔFCR1-c	pCAMBIA0380-Hyg_cassette-OmFCR_del	MUT 68
S288C – empty vector	pFL61 – empty vector	MUT 61
S288C – *Om*FCR1	pFL61-OmFCR1	MUT 59
S288C – *Om*FCR2	pFL61-OmFCR2	MUT 57

For Real-Time quantitative PCR (RT-qPCR), the mycelium was grown on solid Czapek-dox (Oxoid, pH 6) medium, with 1 % agar (Sigma). The mycelium was grown onto sterile cellophane membranes placed on the agar surface. The membranes were prepared by first boiling for 15 min in 10 mM EDTA (disodium salt, dihydrate; Sigma), rinsing, and then autoclaving in ddH_2O. Plates were inoculated with 5 mm plugs of mycelium taken from the edge of a growing fungal colony. *O. maius* Zn isolate was grown on solid medium for 21 d and then transferred on cadmium containing medium (0.4 mM $CdSO_4$; Sigma) for 0.5, 2, 4, 6, 24, 168 h (1 week). Three biological replicates per time-point were prepared. After exposure, the mycelia were peeled off the cellophane membranes and frozen (−80 °C).

To compare growth of *O. maius* Zn wild-type, random mutants and OmFCR1-null mutants, an equal number of conidia were inoculated in 50 mL liquid Czapek mineral medium (pH 4) supplemented with 2 % (w/vol) glucose (Merck) and amended, in the cadmium-exposed samples, with 0.05 mM $CdSO_4$. The mineral medium contained $NaNO_3$ (3 g L^{-1}), $K_2HPO_4 \cdot 3H_2O$ (1.31 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), KCl (0.5 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.01 g L^{-1}). For each condition, three biological replicates were prepared. After 28 d of incubation at 25 °C under constant shaking (120 rpm), the mycelia were filtered and dried for 10 d at RT. Dried mycelia were weighted to evaluate biomass. Statistical analysis was performed by ANOVA with Tukey as *post hoc* test.

Yeast strains and plasmids

All the yeast strains used (Table 1) are derived from EAY1269 (S288C background, genotype MATa *ura3*, *leu2*, *trp1*, *lys2::insE-A14*; Nishant *et al.* 2008) and were kindly provided by Prof. Eric Alani (Cornell University, Ithaca, NY, USA). Yeasts were transformed according to Gietz & Woods (2006), and transformations were confirmed by colony-PCR as described by Sambrook &

Russell (2006), with FL1 and FL2 primers (Table 1S). Amplicons were separated by electrophoresis on 1 % agarose gels. Wild-type yeast cells were grown at 30 °C on Yeast Extract – Peptone medium (YP) supplemented with 2 % (w/v) glucose (YPD). Yeasts transformed with episomes plasmids were grown at 30 °C on Yeast Nitrogen Base (YNB) medium supplemented with 2 % (w/v) glucose and essential amino acids. Transformed yeasts were grown in uracil-dropout medium.

OmFCR2 isolation

Total RNA was extracted from *O. maius* Zn using a CTAB-based protocol (Chang *et al.* 1993; Guether *et al.* 2009). The cDNA was synthesized with Qiagen One Step RT-PCR kit (Qiagen) using primers NotOmFCR2_f and NotOmFCR2_r (Table 1S). Primers were designed to amplify the complete coding sequence of the target genes and to generate *Not*I restriction sites at both ends of the amplicon. The PCR program was as follows: 30 s at 98 °C for one cycle; 30 s at 98 °C, 30 s at 60 °C, 60 s at 72 °C for 35 cycles; 10 min at 72 °C for one cycle. The amplified cDNA was cut with *Not*I and ligated into the pFL61 vector plasmid, previously digested with the same enzyme.

Molecular cloning in *Escherichia coli* was performed *via* electroporation with Bio-Rad MicroPulser™ Electroporator (Bio-Rad), and the plasmid was recovered from transfected colonies with QIAprep Spin Miniprep Kit (Qiagen). Sequencing with primers FL1 and FL2 (Table 1S), designed before and after the MCS, was performed to assess direction of insertion and to confirm cDNA sequence.

Spot dilution assays

Spot dilution assays were used to investigate cell growth on cadmium-amended media. For all conditions, overnight yeast cultures were diluted to OD_{600} = 0.1 and cultured at 30 °C until starting exponential growth (OD_{600} = 0.2). At this stage, 5 µl of serial dilutions (5×10^7 to 5×10^4 cells mL^{-1}) of each strain were spotted on control and cadmium containing YNB media, and incubated at 30 °C for 4 d. All assays were performed with three biological replicates.

RT-qPCR

Total RNA was purified according to a standard protocol (Chang et al., 1993 and Guether et al., 2009). RNA was DNAse treated (Ambion Turbo DNA-free) and cDNA was obtained (from 500 ng of RNA per sample) by using random primers and Superscript II Reverse Transcriptase (Invitrogen). All the primers used for RT-qPCR are listed in Table 1S.

We tested three candidate housekeeping genes, encoding β-tubulin (β-tub), elongation factor 1-α (EF1-α) and triose phosphate isomerase (TIM). The expression data analysis performed by Normfinder (Andersen *et al.* 2004) suggested that the more stable expression profile derived from the average between β-tubulin and elongation factor 1-α expression values (stability value 0.028), compared to the single gene stability values (β-tub 0.031, EF1-α 0.035, TIM 0.037). The β-tub/EF1-α combined expression values were used to calculate relative expression of the genes of interest. RT-qPCR was conducted in a 15 µl reaction mixture containing 7.5 µl of SYBR Green Master Mix (Bio-Rad), 300 nM of forward and reverse primers, and 1 µl of the cDNA template. The qPCR program was: 90 s at 95 °C for one cycle, 15 s at 95 °C and 30 s at Tm° for 40 cycles. At least three technical replicates were performed for all RT-qPCR amplifications. Relative expression of OmFCR1 and OmFCR2 was calculated *via* the 2ˆ-ΔΔCt method (Livak & Schmittgen 2001). The expression values obtained from untreated mycelia (*i.e.* 0 h of Cd exposure) were used as reference. Statistical analysis was performed by ANOVA with Tukey as *post hoc* test.

Knock-out of OmFCR1

The disruption cassette was designed to interrupt the *OmFCR1* gene at position +150 and cause the deletion of a 21 bp-fragment within the coding sequence. The *OmFCR1* disruption cassette carried the 796 bp-5′ *OmFCR1* flanking region (from position −642 to position +154) and the 781 bp-3′ *OmFCR1* flanking region (from position +175 to position +956) amplified from the genomic *O. maius* Zn DNA, separated by the hygromycin resistance expression cassette containing the *Aspergillus nidulans* gpdA promoter, the *Escherichia coli* hygromycin phospho-transferase gene (*hph*), and the *A. nidulans* trpC gene terminator region derived from the pAN7-1 plasmid (Punt *et al.* 1987). Two PCR reactions were set up to generate the flanking regions of the disruption cassette. PCR reactions were carried out in a final volume of 50 µl containing 200 µM of each dNTP, 5 µM of each primer, 5 µl 5× Phusion HF Buffer and 0.5 units of Phusion High-Fidelity DNA Polymerase (Finnzymes). The PCR program was as follows: 30 s at 98 °C for one cycle; 10 s at 98 °C, 45 s at 60 °C, 45 s and 30 s (for each kbp) at 72 °C for 35 cycles, 10 min at 72 °C for one cycle. The 5′- and 3′-flanking regions of OmFCR1 were amplified with primers KO1/KO2 and KO3/KO4 respectively (Table 1S).

The hygromycin-resistance cassette of the pAN7-1 plasmid was digested with *Bgl*II and *Hind*III and inserted within the same restriction sites of the pCAMBIA0380 plasmid. This plasmid was used to generate random mutant strains of *O. maius* Zn. Then, the *OmFCR1* flanking regions were sequentially cloned into the vector, using the *Bgl*II site for the 3′ *OmFCR1* flanking region and the *Xma*I/*Hind*III sites for the 5′ *OmFCR1* flanking region. The correct orientation of the 3′ *OmFCR1* flanking region with respect of the 5′ *OmFCR1* flanking region and the proper construction of the disruption cassette were confirmed by DNA sequencing.

OmFCR1-null mutants as well as random mutants were generated following the *Agrobacterium tumefaciens*-mediated transformation protocol described by Martino *et al.* (2007). To confirm effective knock-out mutations, genomic DNA extracted from transformed mycelia was tested with PCR, RT-PCR and Southern blot using two separate digestions with *Hind*III and *Bgl*II restriction enzymes probed with an *OmFCR1*-specific amplicon and a hph-specific amplicon as previously described (Abbà *et al.* 2009, data not shown).

Bioinformatics

PLAC8 sequences from *Leotiomycetes* were extracted from PFAM and JGI databases (Finn *et al.* 2014). Phylogenetic tree was realized using MAFFT for sequence alignment and FastTree for treebuilding, while for images in Fig 1A and B were used CLUSTAL OMEGA and GSDS (Katoh et al., 2005, Guo et al., 2007 and Price et al., 2009). The phylogenetic tree in Fig 1C was submitted to Treebase (http://treebase.org/treebase-web/home.html) with the submission ID 15256.

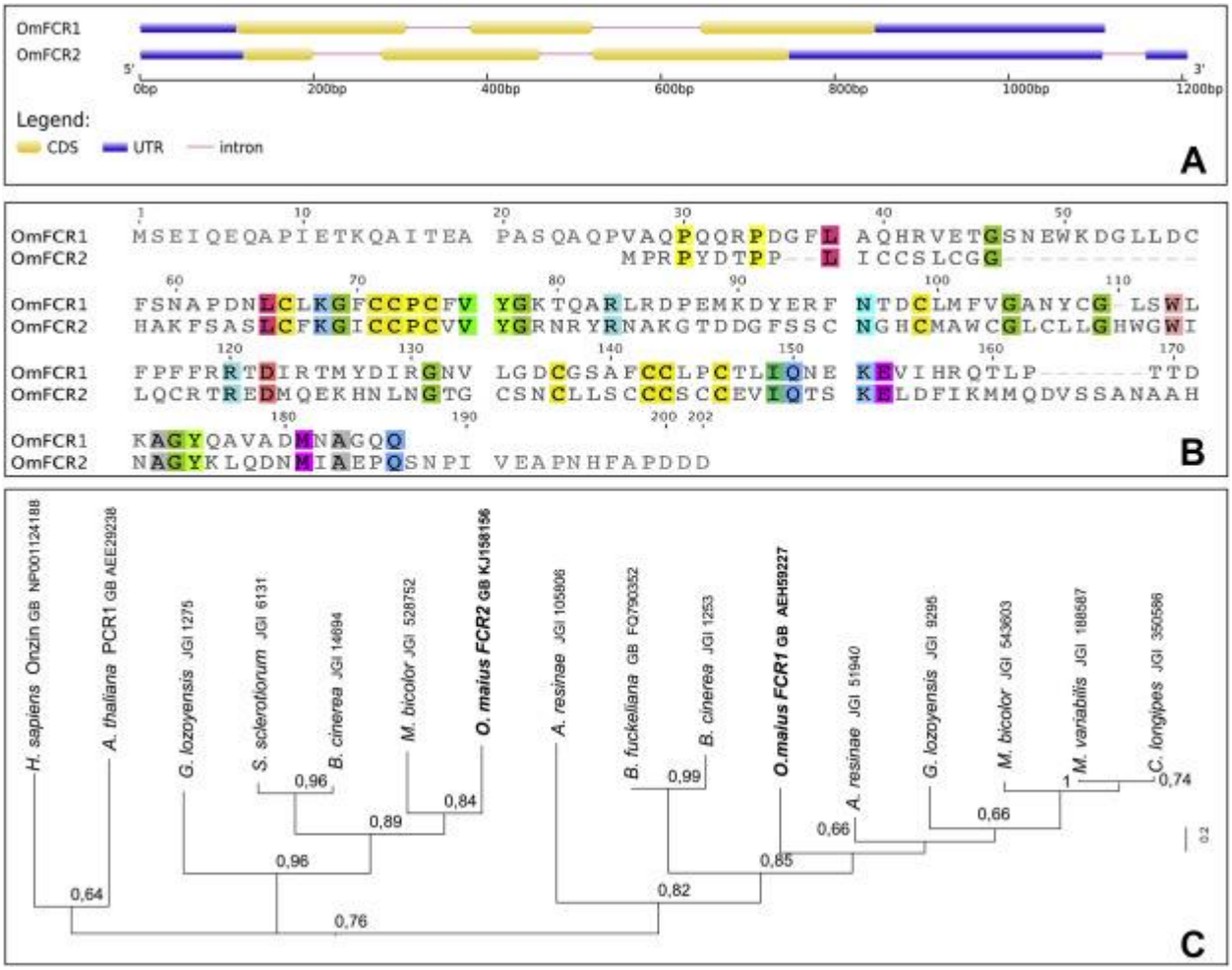

Fig 1.

Gene and deduced protein structure of *OmFCR1* and *OmFCR2*.(A) *OmFCR1* and *OmFCR2* gene models, based on DNA and mRNA sequences. (B) Clustal OMEGA alignment of the OmFCR1 and OmFCR2 amino acid sequences. Amino acids identical between the sequences are highlighted in the same color. (C) Phylogenetic tree of PLAC8-containing protein from species belonging to *Leotiomycetes* genus. *Arabidopsis thaliana* PCR1 and human Onzin PLAC8 genes were inserted as outgroup. Support values are shown if higher than 0.5. *JGI* acronym marks sequences ID from Doe Joint Genome Institute and *GB* acronym marks NCBI Gene Bank sequences ID. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Results

OmFCR2 identification and phenotype in yeast

The complete deduced protein sequence of OmFCR1 (GenBank accession number AEH59227) was used as query in a BLASTp search against the annotated genome of *O. maius* Zn, available on the DOE Joint Genome Institute (JGI) web site (Grigoriev *et al.* 2012). A gene in the *O. maius* Zn genome was identified as coding for a putative protein with *E*-value 2.21E-8 and was named *OmFCR2* (GenBank accession number AHW48112.1).

The protein coded by *OmFCR2* was about the same size as OmFCR1 (163 amino acids) and contained the same PLAC8 (PFAM code PF04749) domain.

CLUSTAL OMEGA alignment of the protein sequences (Sievers & Higgins 2014) showed that the two paralogous proteins share 18.3 % identity, with 37 identical positions and 46 similar positions, in particular the CCPC and CCXXC motifs typical of the PLAC8 domain (Fig 1B). The mRNAs sequences from the *O. maius* Zn transcriptome (also available on the JGI web site) have similar lengths and both feature three exons (Fig 1A). The major difference between the two aminoacidic sequences is in the N-terminal region outside the PLAC8 domain. It is also interesting to observe that putative Metal Responsive Element (MRE) sequences can be found in the promoters of both genes (–TGCACAC–, at position −389 for *OmFCR1* and –TGCCCTC–, at positions −403 and −216 from starting codon for *OmFCR2*) suggesting their role in response to heavy metals.

A comparison between *Leotiomycetes* PLAC8-deduced sequences retrieved from the PFAM and JGI databases (Fig 1C) shows that many of the species analyzed also have two PLAC8 proteins clustering either with OmFCR1 or OmFCR2.

When expressed in the yeast strain S288C, OmFCR2 was able to increase cadmium resistance, but less effectively than OmFCR1, with a maximum tolerated concentration of 10 µM $CdSO_4$ (Fig 2). OmFCR2 could confer cadmium resistance also to yeast mutants lacking YAP1 (data not shown), thus indicating that OmFCR2, similarly to OmFCR1, is implicated in molecular mechanisms independent from YAP1 functions.

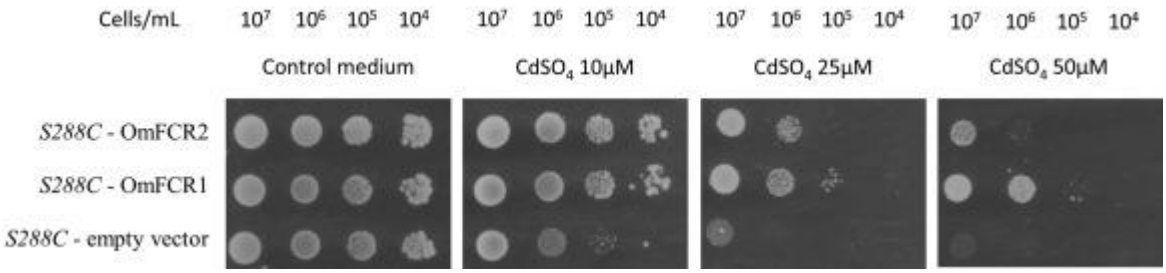

Fig 2.

Sensitivity of *S. cerevisiae* to cadmium. We used yeast expressing OmFCR1, OmFCR2 or an empty vector. Each yeast strain was plated in four serial dilutions on control YNB medium or on YNB with increasing concentrations of $CdSO_4$, as indicated in the figure. Plates were incubated at 30 °C for 4 d.

OmFCR1 and *OmFCR2* expression in *O. maius* Zn

The results of RT-qPCR are summarized in Fig 3, and revealed a different expression profile for *OmFCR1* and *OmFCR2* when the fungal mycelium was exposed to cadmium stress. In particular, *OmFCR1* expression significantly increased 24 h after cadmium exposure, whereas the amount of *OmFCR2* mRNA did not significantly change throughout the experiment.

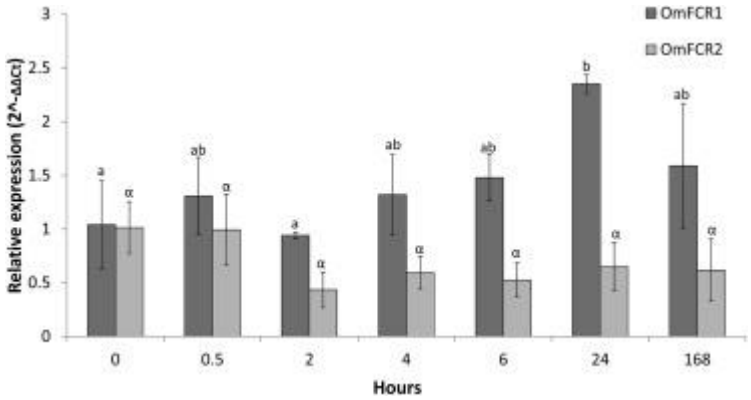

Fig 3.

Gene expression in *O. maius* Zn. *OmFCR1* (dark grey) and *OmFCR2* (light grey) expression was measured by RT-qPCR. The average expression of β-tubulin and elongation factor genes was used to calculate the relative expression values. Samples showing statistically different expression ($p < 0.05$ by ANOVA with Tukey as *post hoc* test) are indicated by different letters (latin letters for *OmFCR1* and greek letters for *OmFCR2*).

Phenotype of the OmFCR1-null mutants of *O. maius* Zn

As the results of RT-qPCR indicated that only *OmFCR1* was induced upon cadmium exposure, we generated knock-out mutants for this gene in *O. maius* Zn. Disruption of the *OmFRC1* gene in all the *O. maius* Zn mutant strains selected for further analyses was confirmed by Southern blot and by reverse transcription (RT-PCR, data not shown). In parallel, we also generated a set of random mutants, in which the selection marker for hygromycin resistance was randomly inserted in the genome. Growth of three OmFCR1-null mutants and three random mutants was compared with growth of the wild-type *O. maius* Zn isolate on both control medium and on medium amended with 0.05 mM Cd. After 30 d of liquid culture, the mycelia were filtered, dried, and weighted (Fig 4). On control medium, all the mutant strains (OmFCR1-null mutants and random mutants) yielded a fungal biomass not statistically different from the wild-type. In the presence of Cd, by contrast, the same mutant strains all grew statistically less ($p < 0.05$) than the wild-type.

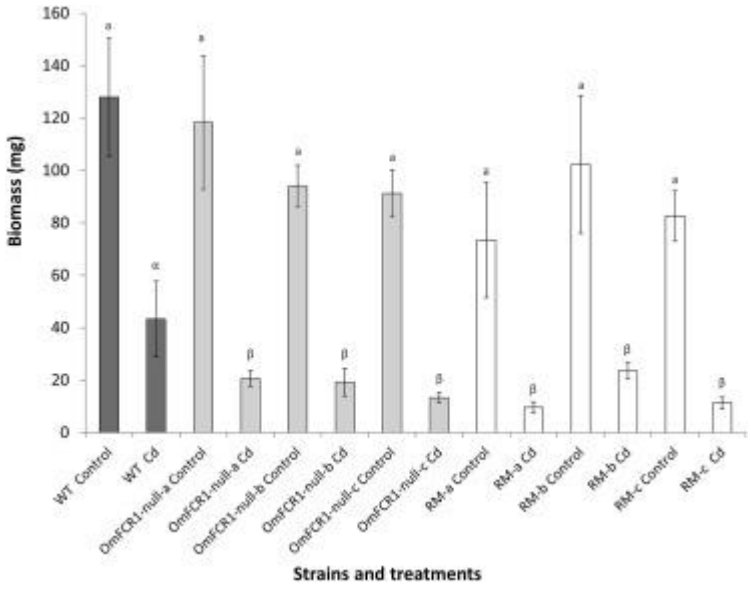

Fig 4.

Dry biomass net weight of *O. maius* Zn strains grown in the absence and in the presence of Cd. *O. maius* Zn wild-type, *OmFCR1*-null mutants and random mutants were grown in control and cadmium-amended medium (0.05 mM). After 30 d the mycelia were filtered and dried. Samples showing statistically different values ($p < 0.05$ by ANOVA with Tukey as *post hoc* test) are indicated by different letters (latin letters for control samples and greek letters for Cd-treated samples).

Discussion

The defense response of fungi to heavy metals mainly relies on avoidance, sequestration and antioxidative mechanisms (Gadd 2010). Our knowledge on the molecular bases of metal tolerance in mycorrhizal fungi is rather limited, but recent studies have contributed to identify genes involved in metal homeostasis. Metal transporters, vacuole-targeting protein, antioxidant enzymes, chaperone proteins, cell-wall proteins, bZIP transcription factors were identified as involved in the general response of mycorrhizal fungi to heavy metals (Jacob et al., 2004, Bellion et al., 2006, González-Guerrero et al., 2010, Bolchi et al., 2011, Ruytinx et al., 2011, Majorel et al., 2012 and Perotto et al., 2012).

OmFCR1 mediates in yeast a specific response to cadmium stress (Abbà *et al.* 2011) that relies on its interaction with MLH3, a subunit of the mismatch repair system, a major complex involved in DNA damage repair (Brown *et al.* 2013). To our knowledge, OmFCR1 was the only member of the PLAC8-containing protein family so far investigated in fungi. Despite the wide distribution of the PLAC8 domain in the eukaryotic kingdoms and the heterogeneous functions found in plants and animals (Libault & Stacey 2010), organisms lacking PLAC8-containing proteins are not rare (*e.g. Saccharomyces cerevisiae* and most invertebrates). This would indicate that proteins containing this specific domain do not play essential cellular functions, or that they have been replaced in some taxa with proteins playing similar functions. The evidence that OmFCR1 is able to interact with yeast cell components (Mlh3p and Dun1; Abbà *et al.* 2011) and to establish a phenotype suggests that, even if the protein is lost in yeast, there is still a molecular network able to recognize it.

The *O. maius* Zn genome features two PLAC8-containing genes that share many features: similar size and gene organization and amino acid sequence similarity of the translated proteins (mainly localized in the PLAC8 domain). Despite their similarities, OmFCR1 and OmFCR2 proteins clustered separately when aligned with PLAC8-containing proteins from other *Leotiomycetes*, suggesting that a gene duplication event occurred in these fungi. Both OmFCR1 and OmFCR2 conferred cadmium tolerance when expressed in yeast, although OmFCR1 was more effective and allowed yeast growth up to 50 μM Cd, whereas OmFCR2 expression resulted in a mildly tolerant phenotype. Both genes were expressed in *O. maius* Zn, but *OmFCR1* expression was significantly induced after 24 h exposure to cadmium, whereas *OmFCR2* expression was low and relatively stable over time. These observations could justify the fact that only *OmFCR1* was originally identified in the yeast functional complementation screening of the *O. maius* Zn cDNA library generated after exposure to cadmium (Abbà *et al.* 2011). It would be interesting to investigate *OmFCR2* expression in the OmFCR1-null mutants, in order to clarify whether it could complement to the absence of *OmFCR1* in Cd-response.

Gene duplication is a powerful source of functional innovation, as paralogous genes derived from duplication events can acquire different molecular and cellular roles (Wapinski et al., 2007 and Gabaldón and Koonin, 2013). In the absence of functional divergence, paralogy may still contribute to modulation of protein dosage or of temporal/spatial expression (Gabaldón & Koonin 2013). We currently do not know if the differences in gene expression and yeast assays observed for *OmFCR1* and *OmFCR2* mirror the acquisition of a new function for one of the two paralogs in *O.*

maius Zn. In any case, the presence of MREs in the promoter regions of both *OmFCR1* and *OmFCR2* would indicate that, although we cannot clearly point to the ancestral gene, metal-mediated induction was an early requirement that has been conserved after duplication. MRE *cis*-acting elements are DNA sequences first identified by Thiele (1992) in metallothionein gene promoters. The MRE sequences found in *OmFCR1* and *OmFCR2* are identical to those reported, in animal cells, in the promoters of several genes targeted by the same zinc-finger transcription factor MTF-1, a major component in heavy metal detoxification (Günther *et al.* 2012). The same MRE consensus sequence was also found in the *poxc* and *poxa1b* laccase gene promoters – both induced by copper – of the fungus *Pleurotus ostreatus* (Faraco *et al.* 2003).

The striking cadmium resistant phenotype conferred by OmFCR1 in yeast (Abbà *et al.* 2011; this study) and the *OmFCR1* transcription induced after cadmium stress in *O. maius* Zn would suggest a direct role of this PLAC8 domain-containing protein in cadmium tolerance. It was therefore quite surprising to observe that growth of the OmFCR1-null mutants on cadmium-amended medium was not statistically different from the growth of three random mutants, at least under the experimental conditions tested.

This result would suggest that OmFCR1 activity in *O. maius* Zn, differently from yeast, is dispensable when the fungus is exposed to cadmium. It should be however noted that *O. maius* Zn is an organism highly tolerant to different heavy metals, and several cellular responses may be activated that allow this fungus to withstand metal exposure. Previous investigations on *O. maius* Zn have shown a possible role of polygalacturonase enzymes (Martino *et al.* 2000) and released organic acids (Martino *et al.* 2003) in defense against heavy metals, as well as the involvement of two membrane metal transporters (Khouja *et al.* 2013) and a Cu/Zn-SOD (Vallino et al., 2009 and Abbà et al., 2011).

Work currently in progress in our lab showed that OmFCR1 expression in yeast significantly reduces the mutation frequency in homonucleotides runs after cadmium exposure, when compared to the same yeast strain expressing the empty vector (L.D., unpubl. data). Therefore, the main function of OmFCR1 in *O. maius* Zn may be in the repair of DNA damage caused by cadmium exposure, likely through its physical interaction with the MLH3 subunit of the mismatch repair system (Abbà *et al.* 2011). This specific role of OmFCR1 in metal tolerance may make measurement of mycelial growth in a short-term experiment not sensitive enough to reveal differences between wild-type and OmFCR1-null mutants.

Conclusions

In conclusion, our results provide new insights on two fungal PLAC8-containing proteins in the metal tolerant ericoid mycorrhizal fungus *O. maius* Zn. OmFCR1 and OmFCR2 likely play different roles in the response of *O. maius* Zn to cadmium, as *OmFCR2* expression was not induced by metal exposure, at least under the experimental conditions tested. Although further studies are required to better understand the origin of these PLAC8-containing proteins and their role in metal tolerance, these cell components contribute to increase the complexity of the heavy-metal response pathways in the ericoid mycorrhizal fungus *O. maius* Zn.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:

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