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Identification par approches moléculaires de gènes impliqués dans la tolérance au stress oxydatif chez le champignon mycorhizien *Oidiodendron maius*

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Dedications

To my parents Mustapha & Labiba…

To my love ziza…

To my brother Mohamed Khairallah, his wife Olfa & their Princess Yasmine…

To my sister Esma Takwallah, her husband Karim & their Princes Yassouna and Ilyes…

To my sister Essia Labiba…

To my grandparents Abdel Hakim & Fatma…
Hassine & Ftouma…
Mohamed & Essia…

To my uncles aunts & cousins…

To my parents Ridha & Sara &
my brothers Ahmed & Haythem…

To all my family…

To all my friends and colleagues…

Hassine Radhouane

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KHOUJA Hassine Radhouane

Molecular approaches to study oxidative stress tolerance mechanisms in the ericoid mycorrhizal fungus *Oidiodendron maius*

Abstract

Due to increasing anthropogenic activities, large areas are highly contaminated by heavy metals which are affecting biological systems. *Oidiodendron maius* strain Zn could be an interesting organism in a bioremediation program being both an ericoid mycorrhizal fungus and a heavy metal-tolerant strain. To understand the mechanisms underlying the oxidative stress tolerance of this strain, three different approaches were used.

The first approach allowed us to obtain superoxide dismutase 1 (SOD1) null mutants. The most important technical advance in this work was the first successful disruption of a gene by homologous recombination in a mycorrhizal fungus. We demonstrate that the lack of *OmSOD* may cause an imbalance in the redox homeostasis and an alteration in the delicate dialogue between the fungus and its host plant.

The second approach was based on a yeast functional complementation screening using an *O. maius* cDNA library. In this work we report the first transporters of an ericoid mycorrhizal fungus capable of conferring Zn tolerance to yeast transformants. Two full-length cDNAs were isolated and named *OmCDF* and *OmFET*. The heterologous expression of these two genes in various yeast mutants conferred resistance to zinc. Additionally, *OmCDF* expression also conferred Co tolerance. We provide evidence that OmCDF functions as a Zn transporter responsible for relocating cytoplasmic Zn into the endoplasmic reticulum, whereas expression of *OmFET* could counteract Zn toxicity by increasing Fe content of cells.

The third approach consisted in the screening of a collection of *O. maius* random-mutants on Zn, Cd and menadione. We report the characterization of an *O. maius*-mutant that carries a mutation in the *nmr* gene. In this mutant, a decrease of glutamine and asparagine pools, and a reduction of the activity of glutamine synthase were recorded. Possible links between the oxidative stress tolerance and the nitrogen metabolism are discussed.

Keywords: ericoid mycorrhizal fungus, tolerance mechanisms, heavy metals, oxidative stress.

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Identification par approches moléculaires de gènes impliqués dans la tolérance au stress oxydatif chez le champignon mycorhizien *Oidiodendron maius*

Résumé
En raison des activités anthropiques croissantes, de larges sites sont contaminés par les métaux lourds qui affectent les systèmes biologiques. La souche *Oidiodendron maius* Zn pourrait être un organisme intéressant dans un programme de bioremédiation étant à la fois un champignon mycorhizien éricoïde et une souche tolérante aux métaux lourds. Pour comprendre les mécanismes de la tolérance de cette souche, trois approches différentes ont été menées.
La première approche a abouti à la génération de mutants du gène superoxyde dismutase 1 (*OmSOD1*). Il s'agit de la première délétion d'un gène par recombinaison homologue chez un champignon mycorhizien. Nous démontrons que l'absence d'*OmSOD1* cause un déséquilibre dans l'homéostasie rédox et un changement dans le dialogue entre le champignon et sa plante hôte.
La deuxième approche a été basée sur la complémentation fonctionnelle d'un mutant de levure en utilisant une banque d'ADNc d'*O. maius*. Nous décrivons les premiers transporteurs d'un champignon mycorhizien éricoïde capables de conférer la tolérance au Zn dans des levures. Deux gènes ont été isolés et nommés OmCDF et OmFET. L'expression hétérologue de ces deux gènes dans différents mutants de levure a permis de conférer la tolérance au Zn. De plus, *OmCDF* a également permis de conférer la tolérance au Co. Nos données suggérent que OmCDF est un transporteur de Zn responsable du transfert du Zn cytoplasmique vers le réticulum endoplasmique, tandis que l'expression d'OmFET pourrait neutraliser la toxicité engendrée par le Zn en augmentant le contenu du Fe dans la cellule.
La troisième approche a concerné le criblage d'une collection de mutants aléatoires d'*O. maius* sur Zn, Cd et ménadione. Nous décrivons la caractérisation d'un mutant dans le gène *nmr*. Dans ce mutant, une diminution de la teneur en glutamine et asparagine, ainsi qu'une réduction de l'activité de la glutamine synthétase ont été enregistrées. Les liens possibles entre la tolérance au stress oxydatif et le métabolisme azoté sont discutés.

Mots clés : champignon mycorhizien éricoïde, mécanismes de tolérance, métaux lourds, stress oxydatif.

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Résumé de la thèse

En raison des activités anthropiques croissantes, de larges surfaces sont fortement contaminées par les métaux lourds qui affectent le système biologique. La souche *Oidiodendron maius* Zn pourrait être un organisme intéressant dans un programme de bioremédiation car il est à la fois un champignon mycorhizien éricoïde et une souche tolérante aux métaux lourds. Cette souche a été isolée à partir des racines de *Vaccinium myrtillus* cultivés dans des sites expérimentaux fortement contaminés par des concentrations élevées de zinc, de cadmium et d'aluminium. La souche *O. maius* Zn a montré une tolérance élevée aux métaux lourds, particulièrement au zinc. Cette souche s'avère donc très intéressante pour l'étude des mécanismes de tolérance aux métaux lourds et pour la compréhension du rôle de ce champignon symbiotique dans la protection de sa plante hôte. Pour comprendre les mécanismes qui sont à la base de la tolérance au stress oxydatif, et plus généralement aux métaux lourds, de cette souche, trois approches différentes ont été menées.

La première approche nous a permis d'obtenir la génération de mutants du gène superoxyde dismutase 1 (*OmSOD1*). Un des faits marquants de ce travail était la première délétion réussie d'un gène par recombinaison homologue chez un champignon mycorhizien. La transformation génétique par *Agrobacterium tumefaciens* a en effet été réalisée avec succès pour la délétion ciblée de l'unique copie du gène *OmSOD1*. Etant à la fois un champignon mycorhizien et une souche tolérante aux métaux lourds, nous avons testé si l'absence du gène *OmSOD1* d'*O. maius* pourrait ; d'une part, altérer la capacité d'établir des symbioses avec les racines de sa plante hôte ; et d'autre part, influencer la réponse du champignon au stress oxydatif. Parmi les mutants obtenus trois ont été choisis pour évaluer, en comparaison avec la souche sauvage, leurs croissances en présence de zinc, de cadmium ou de ménadione, le pourcentage de formation de conidies, et la capacité d'établir des symbioses avec la plante hôte. La délétion d'*OmSOD1* a abouti à la présence de marqueurs de stress oxydatif, même en absence de stress externes et une sensibilité accrue aux substances sources de stress oxydatif. Une réduction de la formation des conidies, ainsi que du pourcentage de mycorhization avec les racines de *Vaccinium myrtillus* a également été observée. Ainsi, nous suggérons que l'absence de cette protéine puisse causer un déséquilibre dans l'homéostasie redox pendant la colonisation de l'hôte et ainsi induire un changement dans le dialogue entre le champignon et sa plante hôte.

La deuxième approche a été basée sur la complémentation fonctionnelle d'une souche mutante de *Saccharomyces cerevisiae* Δ*zrc1* (sensible au zinc) en utilisant une banque d'ADNc d'*O. maius* Zn. Dans ce travail nous décrivons les premiers transporteurs impliqués dans la tolérance au zinc chez un champignon mycorhizien éricoïde. Deux gènes ont ainsi été

identifiés par complémentation de la souche Δ*zrc1* : (1) un membre de la famille des transporteurs CDF (Cation Diffusion Facilitator), nommé *OmCDF* ; et (2) un membre de la famille des perméases du fer, nommé *OmFET*. Des études plus approfondies ont été effectuées pour évaluer la spécificité de la tolérance aux métaux et la localisation subcellulaire de ces protéines. L'expression hétérologue d'*OmCDF* et *OmFET* dans différents mutants de levure a permis de conférer la résistance au zinc. De plus, l'expression d'*OmCDF* a permis de conférer également la tolérance au cobalt. La localisation de la fluorescence issue de la protéine chimérique OmCDF::GFP a été observée au niveau de la membrane du réticulum endoplasmique, tandis que celle d'OmFET::GFP a été retrouvée au niveau de la membrane plasmique. Nous avons mis en évidence la fonction d'OmCDF comme étant un transporteur de zinc responsable du transfert du zinc cytoplasmique vers le réticulum endoplasmique, tandis que l'expression d'OmFET pourrait neutraliser la toxicité engendrée par le zinc en augmentant le contenu du fer dans la cellule.

La troisième approche a porté sur le criblage d'une collection de 1087 mutants aléatoires de la souche *O. maius* Zn en présence de zinc, de cadmium ou de ménadione afin d'identifier des mutants hypersensibles. Il s'agit du premier travail illustrant un criblage de mutants aléatoires, avec identification des gènes mutés, effectué chez un champignon mycorhizien. Les mutants ont été obtenus à partir de transformations de conidies de la souche *O. maius* Zn afin de définir la fonction de gènes spécifiques impliqués dans le mécanisme de tolérance au stress oxydatif. Parmi les 309 mutants criblés, 23 ont présenté au niveau phénotypique une sensibilité plus élevée que celle de la souche sauvage, et ce au moins envers une des substances testées. L'analyse moléculaire par TAIL-PCR « Thermal Asymetric Interlaced-PCR » a permis d'identifier trois gènes : un membre de la famille des facilitateurs majeurs (MFS), un facteur de transcription bZIP et un gène de « répression métabolique azotée » (*nmr*). Parmi les candidats, nous avons caractérisé le phénotype du mutant *OmNmr1*. La concentration en acides aminés libres, ainsi que les activités de la glutamine synthétase (GS) et de la glutamate déshydrogénase (GDH) de la souche sauvage et du mutant ont été évaluées. Dans le mutant, une augmentation de la quantité de glutamate et d'alanine et une diminution de la teneur en glutamine et en asparagine ont été enregistrées. L'activité de la GS est significativement réduite dans le mutant et est compensée par l'activité de la GDH. Cette réduction de l'activité de la GS pourrait expliquer la diminution de la quantité de glutamine et d'asparagine dans le mutant. La biomasse du mycélium du mutant est plus importante sur le milieu contenant de la glutamine, comparativement aux milieux contenant d'autres sources d'azote. La faible production de glutamine, ainsi que la faible activité de la GS pourrait influencer la biosynthèse du glutathion, expliquant ainsi la forte sensibilité du mutant au zinc,

au cadmium et à la ménadione. Ce mutant représente un outil très intéressant pour l'étude du métabolisme azoté et la tolérance au stress oxydatif dans le champignon éricoïde *O. maius*.

L'ensemble de ces approches nous a permis d'acquérir des connaissances approfondies sur l'homéostasie des métaux et la tolérance au stress oxydatif chez le champignon mycorhizien *O. maius*.

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Chapter 1

GENERAL INTRODUCTION

1. Heavy metals in the environment: Sources, Toxicity and Remediation techniques

1.1. Sources

A heavy metal is a member of a loosely-defined subset of elements that exhibit metallic properties. It mainly includes the transition metals, some metalloids, lanthanides and actinides (Fig. 1). Heavy metals originate from natural sources such as rocks and metalliferous minerals, and also from anthropogenic inputs. Due to increasing anthropogenic activities such as industry, agriculture, mining, transportation, construction and habitations, heavy metals can become locally concentrated and are becoming widespread environmental contaminants all over the world (Lehoczky *et al.*, 2004; Mehradadi *et al*, 2006; Kozlov and Zvereva, 2007; Liao *et al.*, 2008). One of the most crucial properties of heavy metals is that they are not biodegradable (Rauret *et al.*, 1999), and they will have permanent effect on organisms in contaminated areas.

Thirteen trace metals and metalloids (Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl and Zn) are considered priority pollutants (Sparks, 2005). Metals have increasingly been redistributed in the environment, with accumulation in terrestrial and aquatic habitats. Atmospheric deposition is a major mechanism for metal input to the environment. Volatile metal(loid)s such as As, Hg and Sb can be distributed as gases or enriched in particles, while Cu, Pb and Zn are transported as particulates (Adriano, 2001; Adriano *et al.*, 2004).

Many metals are essential for life, e.g. Na, K, Cu, Zn, Co, Ca, Mg, Mn and Fe, but all can exert toxicity when present above certain threshold concentrations. Other metals, e.g. Cs, Al, Cd, Hg and Pb, have no known essential metabolic functions but all can be accumulated (Gadd, 2010). Although Cd is considered as a non essential metal, Lane and Morel (2000) provided evidence of a biological role for Cd as a cofactor of a Cd-specific carboanhydrase in the marine diatom *Thalassiosira weissflogii* under conditions of low zinc, typical of the marine environment.

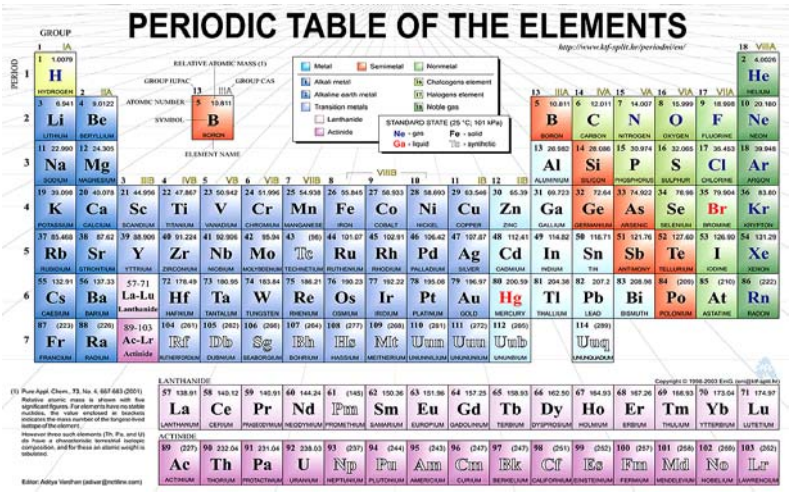

Figure 1. Periodic table of the elements. Metal elements are in blue, semi-metal (metalloid) elements are in orange and non-metal elements are in green (http://blogs.4j.lane.edu/taylor_k/2010/06/01/metals-this-week-in-science-june-1-june-4/).

1.2. Metal toxicity mechanisms

In small quantities, certain heavy metals are nutritionally essential for a healthy life, and some are reported as trace elements such as Fe, Cu, Mn and Zn. However, when present in excessive amounts, they affect the biological systems.

According to their physical and biochemical properties, different molecular mechanisms of metal toxicity can be distinguished: (i) production of reactive oxygen species (ROS), mainly for redox-active metals; (ii) inhibition of protein function by blocking of functional groups especially for redox-inactive metals and by displacement of essential metal ions; and (iii) impaired DNA repair pathways (Fig. 2).

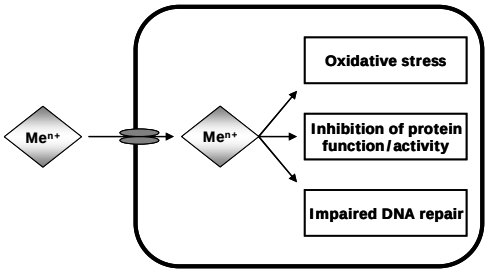

Figure 2. Metal toxicity mechanisms in living organisms at cellular level (Modified from Wysocki and Tamás, 2010)

1.2.1. Heavy metals and oxidative stress

Many studies have shown that several heavy metals exhibit the ability to produce reactive oxygen species (ROS), generating oxidative stress. ROS are mainly singlet oxygen (O^-), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). ROS can damage all cellular macromolecules, leading to protein oxidation, lipid peroxidation and DNA damage (Halliwell and Gutteridge, 1984).

Fe, Cu, Cr and Co can undergo redox-cycling reactions, while Cd, Hg, Ni, Zn and Pb are redox inactive metals. Formation of metal-induced ROS can occur via several mechanisms. The Fenton or Haber-Weiss reactions are catalyzed by redox-active metals and generate the highly reactive hydroxyl radical from H_2O_2 and superoxide substrate. In the Fenton reaction, the peroxide is broken down into a hydroxide ion and a hydroxyl free radical. The hydroxyl free radical is the primary oxidizing species and can be used to oxidize and break apart organic molecules. The Haber-Weiss reaction generates hydroxyl radicals from hydrogen peroxide and superoxide (Halliwell and Gutteridge, 1999).

The redox-inactive metals may induce oxidative stress by inhibiting specific enzymes, by depleting pools of antioxidants or through other indirect mechanisms (Stohs and Bagchi, 1995; Ercal *et al.*, 2001; Beyersmann and Hartwig, 2008). For example, redox-inactive metals may perturb intracellular Fe metabolism, leading to increased levels of free Fe in the cell, which in turn could enhance Fenton-type reactions and elevated ROS levels (Kitchin and Wallace, 2008).

An example of the effect of redox-inactive metals on the antioxidants pools is the depletion of glutathione (GSH), which is the main antioxidant in cells (Stohs and Bagchi, 1995). GSH depletion would influence the redox system and damage the activities of GSH-dependant enzymes such glutathione peroxidases, glutathione S-transferases and glutaredoxins, thereby affecting many cellular processes (Wysocki and Tamás, 2010).

In the ectomycorrhizal fungi *P. involutus*, Jacob *et al.* (2001) hypothesized that Cd, although it is a redox-inactive metal, induced an oxidative stress by affecting the cellular thiol redox balance.

1.2.2. Inhibition of protein function and activity

Heavy metals have the capacity to bind proteins, often via thiol groups of cysteine residues, and inhibit enzyme function. The inhibition of proteins involved in the protection of cells from oxidative stress would lead to increased ROS levels.

Cadmium may displace zinc and calcium ions from metalloproteins (Stohs and Bagchi, 1995; Faller *et al.*, 2005) and zinc finger proteins (Hartwig, 2001), thereby damaging their activity. As(III) has been shown to interact with actin, tubulin and thioredoxin reductase and many other proteins (Hoffman and Lane, 1992; Menzel *et al.*, 1999; Zhang *et al.*, 2007; Kitchin and Wallace, 2008). Moreover, Cr exposure has been shown to trigger oxidative protein damage and enhanced protein aggregation in yeast (Summer *et al.*, 2005; Holland *et al.*, 2007).

1.2.3. Impaired DNA repair

Most metals are weak mutagens but they do not damage DNA directly; instead, they may trigger genotoxicity by interfering with DNA repair processes (Beyersmann and Hartwig, 2008). The effect of some metals on the DNA repair systems is reported for yeast. For example, cadmium increases the fixation of mutations resulting from endogenous processes by inhibiting the DNA mismatch repair pathway (Jin *et al.*, 2003). Serero *et al.*, (2008) screened a *S. cerevisiae* deletion collection for mutants sensitive to Cd and identified two genes, RAD27 and DNA2, involved in DNA repair and replication. Theses results suggest that DNA replication is also a target of Cd toxicity. A *S. cerevisiae* mutant affected in the error-prone repair system was sensitive to Se(III), while mutations in the other DNA repair pathways did not strongly affect resistance to Se(III) (Pinson *et al.*, 2000). As(III) can also induce DNA damage in mammalian systems by interfering with DNA repair systems (Shi *et al.*, 2004). However, genes involved in DNA repair in yeast, when exposed to As(III), are not yet identified.

1.3. Remediation techniques

Contamination of soils with heavy metals represents a serious threat for the ecosystem and human health. Several physical, chemical and biological strategies have been developed for the removal of these toxic substances. These strategies may be used in conjunction with each other to reduce the contamination to a safe and acceptable level.

1.3.1. Physicochemical approaches

Chemical methods consist in the solidification, precipitation and ion exchange, and the physical methods consist in the soil washing, encapsulation, and electrokinesis. Chemical remediation involves the use of chemicals to clean the natural environment. However, this approach appears to have some disadvantages as the same chemical cannot be used for all metal ions (Chaney *et al.*, 1997), and the existence of many classes of chemical species makes the removal of the toxic metals from the environment very complicated.

In summary, physicochemical strategies have been developed for removing heavy metals from the polluted soil and water. However, most of them appear to be expensive and with low efficiency.

1.3.2. Phytoremediation

Phytoremediation is the use of plants to remove or detoxify environmental pollutants (Baker and Brooks, 1989; Salt *et al.*, 1998). This technique is considered to be less invasive, more cost-effective and restorative of soil structure. Moreover, some extracted metals may be recycled for value. Phytoremediation can be divided into phytoextraction (pollutant removal from soil into shoots and leaves), phytodegradation (pollutant degradation by plant-microbe systems), rhizofiltration (absorption of pollutants by plant roots), phytostabilization (plant-mediated reduction of pollutant bioavailability), phytovolatilization (plant mediated volatilization of pollutants) and phytoscrubbing (plant removal of atmospheric pollutants) (Gadd, 2010).

Phytoextraction is the extraction of metals from soil and is used to clean-up heavy metals, pesticides, xenobiotics, and other toxic pollutants. Plant ability to phytoextract certain metals is a result of its dependence upon the absorption of metals such as Zn, Mn, Ni and Cu to maintain natural functions (Lasat, 2002). Chelate-assisted and continuous phytoextraction consist in the application of various synthetic chelators that can enhance metal accumulation. This strategy has been developed because plants do not naturally accumulate toxic metals to levels that would be significant in a remediation context (Gadd, 2010). Phytoextraction has attracted attention in recent years for the low cost of implementation and environmental

benefits. However, soils contaminated with multiple heavy metals can be a difficult challenge for phytoextraction. Although, some hyperaccumulating plants appear to be capable of accumulating elevated concentrations of several heavy metals simultaneously, there still remains considerable specificity in metal hyperaccumulation (Baker *et al.,* 2000).

Rhizofiltration is similar to phytoextraction, and plants are essentially used to remove pollutants from contaminated groundwater, but in this case pollutants are normally accumulated at the root level.

Phytostabilization is the use of plant to immobilize contaminants in the soil and groundwater through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants. It is useful for the treatment of Pb, As, Cd, Cr, Cu and Zn. The advantage of this technology is that it is very effective when rapid immobilization is needed to preserve ground and surface waters (Jadia and Fulekar, 2009).

Phytovolatization involves the use of plants to take up contaminants from the soil, transforming them into volatile forms and transpiring them into the atmosphere (Jadia and Fulekar, 2009). The metal can be rendered harmless by either enzymatic reduction or by incorporation into less toxic organic compounds.

Phytoremediation techniques, although being slow if compared to the physico-chemical ones, have the advantages to be less costly and much more environmental friendly.

1.3.3. Bioremediation: the case of Mycoremediation

Bioremediation is the application of biological systems for the removal of organic and inorganic pollution. Bacteria and fungi are the most important organisms for the remediation of heavy metals. Hereafter I will focus on the mycoremediation strategy.

Mycorrhizal fungi are ubiquitous soil microorganisms that associate with the roots of almost all land plants and assist their hosts in nutrient uptake (Smith and Read, 1997). They do so by exploring extensive volumes of soil with their extraradical mycelium, and also by solubilizing essential nutrients that are normally found as insoluble organic and inorganic compounds (Marschner, 1995; Martino and Perotto, 2010; Gadd, 2010). Mycorrhizal associations, including arbuscular, ecto- and ericoid mycorrhizas, are considered as a beneficial strategy in the general area of phytoremediation as they increase plant metal tolerance (Van der Lelie *et al*., 2001; Rosen *et al*., 2005, Gohre and Paszkowski, 2006). Mycorrhizas may both increase and reduce plant metal accumulation, and may enhance phytoextraction directly or indirectly by increasing plant biomass (Tullio *et al*., 2003).

Christie *et al*. (2004) found that arbuscular mycorrhizal fungi (AMF) depressed zinc translocation to shoots of host plants by binding of metals in mycorrhizal structures and by immobilizing metals in the mycorrhizosphere. In addition, AMF produce the insoluble

glycoprotein glomalin that can sequester metals and which could be considered a useful stabilization agent in remediation of polluted soils (González-Chávez *et al.*, 2004). *Glomus intraradices* increased root uranium (U) concentration and content, but decreased shoot U concentrations, when it was associated with *Medicago truncatula* (Chen *et al.*, 2005). Thus, AMF generally decreased U translocation from plant root to shoot (Rufyikiri *et al.*, 2004).

In ectomycorrhizal fungi, Cu-adapted *S. luteus* isolates provided excellent insurance against Cu toxicity in pine seedlings exposed to elevated copper and have been proposed to be suitable for large-scale land reclamation at phototoxic metalliferous and industrial sites (Adriaensen *et al.*, 2005). Krupa and Kozdroj (2004) found that ectomycorrhizal fungi persistently fixed Cd(II) and Pb(II), and formed an efficient biological barrier that reduce the translocation of metals into birch tissues. Such mycorrhizal metal immobilization around plant roots may assist soil remediation and revegetation.

Ericoid mycorrhizal fungi are also reported to alleviate metal toxicity to ericaceous plants such as *Calluna*, *Erica* and *Vaccinium* spp. In metal-polluted soils, the fungus prevents metal translocation to plant shoots (Bradley *et al.*, 1981; 1982). Thanks to the symbiotic association with ericoid mycorrhizal fungi, ericaceous plants are able to grow in highly polluted environments (Perotto *et al.*, 2002; Martino *et al.*, 2003). The adaptation of ericaceous plants to polluted soils is mainly related to the acquisition of toxic metal resistance by the ericoid mycorrhizal fungi (Cairney and Meharg, 2003).

The development of stress-tolerant-mycorrhizal associations may therefore be a promising strategy for soil phytoremediation and amelioration.

2. Mechanisms of metal tolerance in fungi

Several metal ions, such as Zn, Cu and Mn, are essential elements for living organisms, but at high concentrations they become toxic. Other metals that are not essential, such as Cd, Hg and Pb are toxic even at very low concentrations (Trevors *et al.*, 1986; Hall, 2002). Despite the presence of toxic elements a number of microorganisms can grow in contaminated sites, and this could be explained by the development of a wide variety of mechanisms to cope with metal stress.

In fungi, the mechanisms potentially involved in metal tolerance can be classified into three groups: (i) extracellular mechanisms such as chelation and cell-wall binding, which metal entry; (ii) intracellular mechanisms including the metal binding to (non)-protein thiols and transport into intracellular compartments, which reduce metal burden in the cytosol; and (iii) detoxification mechanisms which allow the fungus to counteract ROS accumulation directly

or indirectly (Fig. 3). Some of these mechanisms are constitutively present, whereas others are only activated when metals exceed a threshold value (Colpaert, 2008).

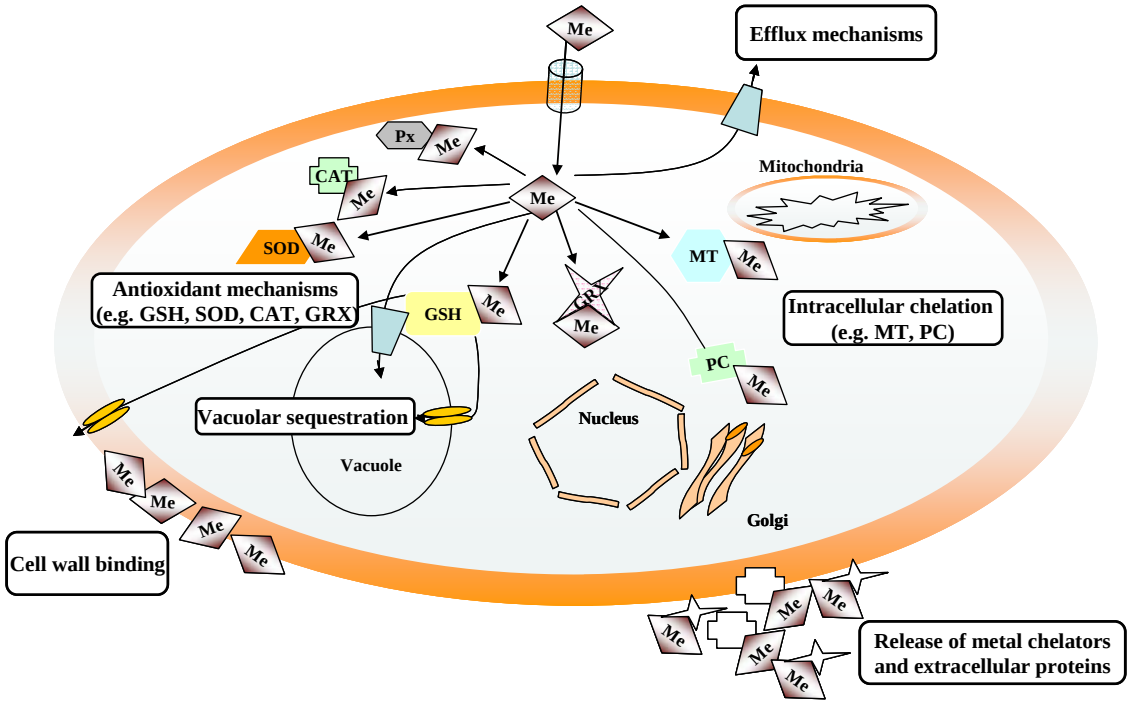

Figure 3. Potential mechanisms involved in heavy metal and oxidative stress tolerance in fungi. Me: Metal; MT: Metallothionein; PC: Phytochelatin; GSH: Glutathione; SOD: Superoxide dismutase; CAT: Catalase; GRX: Glutaredoxin; Px: Peroxidase.

2.1. Extracellular mechanisms

Extracellular mechanisms allow a reduction of metal uptake thanks to the organic acid-chelation and the metal-binding to extracellular proteins.

2.1.1. Chelation

2.1.1.1.Organic acids

Fungi can excrete many primary and secondary metabolites with metal complexing properties such as carboxylic acids and amino acids (Muller *et al.*, 1995). The exudation of organic acids by fungi is a process that can affect metal toxicity. Different organic molecules, such as citric and oxalic acids, can chelate or precipitate metal ions thereby reducing metal uptake into the fungal cells. Given the functional dualism of acidification and chelation (Landeweert *et al.*, 2001), both oxalate and citrate are considered as the most efficient substances that can scavenge metals.

Citric acid has been reported to change the chemical forms of heavy metal by the transformation of toxic forms into less toxic forms. For example, citric acid has been shown to be the most important Al^{3+} complexing agent in soil solution (Landeweert *et al.*, 2001).

Fungi can also produce oxalates that interact with a variety of different metals, e.g. Ca, Cd, Co, Cu, Mg, Mn, Sr, Zn, Ni and Pb (White et al., 1997; Gadd, 1999; Sayer *et al.*, 1999; Sayer and Gadd, 1997; Gadd, 2007). The formation of toxic metal oxalates provides a mechanism enabling fungi to tolerate high concentrations of metal compounds (Gadd, 1993). Oxalic acid interacts with metal ions to form insoluble oxalate crystals around the cell wall and in the external medium (Murphy and Levy, 1983). In the ectomycorrhizal fungi *Pisolithus tinctorius* and *Laccaria bicolor* (Cumming *et al.*, 2001), Al induces oxalate production, although in this case the role of Al chelation in the tolerance mechanism is not clear. On the other hand, the induction of oxalic acid efflux correlated closely with Cu tolerance in brown rot fungi (Green and Clausen, 2003). Similarly, Fomina *et al.* (2005) attributed to the over-excretion of oxalic acid the metal tolerance observed in *Beauveria caledonia*. In *Paxillus involutus*, oxalic acid reduces Cd uptake by more than 85% (Blaudez, unpublished results).

Therefore, according to literature, there is normally a direct correlation between the production of organic acids and the reduction of metal toxicity. However, Martino *et al.* (2003) reported that *O. maius* strains from polluted sites showed a lower organic acid production than isolates from unpolluted sites in the presence of high zinc concentrations. Authors speculated that such a reduction could be an adaptation to metal toxicity and may reflect specific strategies to maintain homeostasis of essential metals under different soil conditions.

2.1.1.2.Extracellular proteins

Many extracellular proteins have theability to interact with metals and are therefore important cell components that bind these elements outside the cell wall.

Glomalin-related soil protein (GRSP), as termed by Rillig (2004), is a glycoprotein excreted by arbuscular mycorrhizal fungi. This protein appears to be a component of the hyphae and spore wall, likely released into the soil by mycelium turnover. GRSP, which plays a key role in soil stability, has been also proposed to be a potential component for the remediation of polluted soil as it is able to sequester potentially toxic elements (González-Chávez *et al.*, 2004; Cornejo *et al.*, 2008). The release and accumulation of GRSP in soils can be a very important mechanism for the stabilization of degraded soils; it may also contribute to sequestration of significant quantities of heavy metals.

In the ericoid mycorrhizal fungus *O. maius* Zn, the presence of high concentrations of zinc in the growth medium induces the production of extracellular superoxide dismutases (SODs) (Martino *et al.*, 2002, Vallino *et al.*, 2009). SODs are known to be central regulators of reactive oxygen species (ROS). Thus, the extracellular SOD enzymes could play an important

role in the defense against the oxidative stress induced by the potentially toxic metal ions. In *O. maius* increasing zinc concentrations also induce the production of some hydrolytic enzymes such as nuclease, proteinase and lysozyme (Martino *et al.*, 2002).

2.1.2. Cell wall binding

Metal retention by the cell wall is reported as an important mechanism for metal detoxification in fungi (Gadd, 1993; Gadd, 2005). The fungal cell wall directly interacts with metals and shows a high capacity for metal binding. This mechanism, termed biosorption (Gadd, 1993), is not depending on the metabolic activity of the fungus. It is a metabolically passive process, consisting on a physico-chemical interaction between the cell wall and metals. Mechanisms of biosorption may include ion exchange, adsorption, complexation, precipitation and crystallization (Bartnicki-Garcia, 1968; Strandberg *et al.*, 1981; Tobin *et al.*, 1990). The diversity of the fungal cell wall composition (glucan-, chitin-, and galactosamine-containing polymers) provides a large number of potential metal binding sites such as carboxyl, amine, hydroxyl, phosphate and mercapto groups (Strandberg *et al.*, 1981). In *Aspergillus niger*, carboxyl groups are reported to be involved in the cell well binding of Cd^{+}, Ag^{+} and CH_3Hg^{+} (Akthar *et al.*, 1995; Karunasagar *et al.*, 2004). Binding of Cd onto cell walls of *Paxillus involutus* is considered as one of the essential metal detoxification mechanisms (Blaudez *et al.*, 2000).

Fungal cell walls are typically composed of the polysaccharides chitin and cellulose. Chitin and chitosan are important components known to bind toxic metal ions. Chitin is a linear polymer constituted of L-1,4-linked N-acetyl-D-glucosamine residues. It is the main component of the cell walls of fungi. Several studies on chitin metal interactions were previously reported. Kurita and colleagues (1979) reported that the chitin is potentially useful for the removal of metals. In *N. crassa*, cell walls accumulated a high percentage of the metal present in the medium (Subramanyam *et al.*, 1983). In an effort to understand the complexation phenomenon of metals to chitin, Bhanoori and Venkateswerlu (2000) were able to attribute to the ring and C-3 hydroxyl oxygens of N-acetylglucosamine the cadmium complexation activity. Lanfranco *et al.* (2002b) reported a significant increase of the chitin amount in an ericoid mycorrhizal fungus, when exposed to metals. Studies on zinc ion biosorption by *R. arrhizus* showed that Zn was predominantly bound to cell-wall chitin and chitosan.

Chitosan, which is a deacetylated derivative of chitin, is an efficient chelating agent. It can be found in fungi, but its occurrence in nature is much less abundant if compared to chitin.

However, chitosan has a high metal binding capacity and chelates five to six times more metals than chitin (Vasconcelos *et al.*, 2008).

The extracellular production of melanin may contribute to metal binding (Fomina and Gadd, 2003). It is well known that fungal melanins have a high biosorptive capacity for a variety of metal ions and the presence of melanin in pigmented cell walls is responsible for high levels of biosorption (Gadd, 1993). Therefore, melanin can enhance the fungal survival in response to environmental stress by further increasing the metal biosorption capacity. For example, the melanin-producing fungus *Cladosporium cladosporioides* has a high sorption capacity for Cd and Cu (Fomina and Gadd, 2003). In addition, a correlation was reported between the production of melanin and metal tolerance in the phytopathogenic *Streptomyces scabies* by analyzing mutants deficient in melanin biosynthesis (Beauséjour and Beaulieu, 2004).

2.2. Intracellular mechanisms

Despite the extracellular chelation and/or cell-wall binding, metals can enter into cells mainly when the mycelium is exposed to high metal concentrations. Two basic strategies to cope with intracellular metal toxicity are known: (i) chelation of metal ions and (ii) metals efflux from the cytosol, either out of cells or into intracellular organelles.

2.2.1. Chelation of metal ions in the cytosol

Chelators are responsible for the buffering of toxic metal concentration within cells. Metallothioneins (MTs) and phytochelatins (PCs) represent the main chelators of metal ions in the cytosol (Cobbett and Goldsbrough, 2002). In addition, both redox-active and redox-inactive metals may increase the production of ROS such as hydroxyl radical (HO^-), superoxide radical (O_2^-) or hydrogen peroxide (H_2O_2). To protect against ROS damages, cells have developed various antioxidant defense systems.

2.2.1.1.Metallothionein (MT)

Metallothioneins (MTs) are a family of cysteine-rich, low molecular weight proteins, found in a wide range of organisms including bacteria, fungi, animal and plants. MTs have the capacity to bind essential and non essential heavy metals through the thiol group of their cysteine residues and they are involved in the homeostasis of metals and also in the detoxification of toxic elements (Bourdineaud *et al.*, 2006). The first evidence for metal tolerance in fungi was the isolation of the "metallothionein (MT)-like" peptide from the ectomycorrhizal fungus *Pisolithus tinctorius* (Morselt *et al.*, 1986). After that, Howe *et al.* (1997) reported the presence of Cu-binding proteins, which had properties characteristic of MTs, in *Laccaria*

laccata and *Paxillus involutus*. MTs had a relatively high specificity to only one or few metal species. In fact, MTs from *Gigaspora margarita* (Lanfranco *et al.*, 2002a), *Candida glabrata* (Mehra *et al.*, 1989), and *Podospora anserina* (Averbeck *et al.*, 2001) responded to Cu, but not to Cd exposure. The *Pimt1* gene, coding for a MT from *P. involutus*, was demonstrated to be a potential determinant in the response to Cu and Cd, but not to Zn stress (Bellion *et al*., 2007). In addition, the expression of the MT gene from the filamentous cyanobacterium *Oscillatoria brevis* increased in the presence of Cd and Zn (Liu *et al.*, 2003).

2.2.1.2.Phytochelatin (PC)

Phytochelatins (PCs) are a family of small cystein-rich peptides capable of binding heavy metal ions through the SH group. PCs are enzymatically synthesized from glutathione and are found in yeasts, algae and plants (Rauser, 1999; Cobbett, 2000; Cobbett and Goldsbrough, 2002). PCs have been widely studied in plants, especially for their implication in the Cd tolerance (Cobbett, 2000; Cobbett and Goldsbrough, 2002). In fungi, only the two yeast *C. glabrata* (Mehra *et al.*, 1989) and *Schizosaccharomyces pombe* (Clemens *et al.*, 1999; Ha *et al.*, 1999) have been reported to synthesize PCs. PC synthase from *S. pombe* (SpPCS) has been expressed in *E. coli*, resulting in higher arsenic accumulation (Tsai *et al*., 2009). The lack of phytochelatins has been reported for several filamentous fungi such as *P. involutus* (Courbot *et al.*, 2004), *O. maius* (Vallino *et al*., 2005), and *Laccaria laccata* (Galli *et al*., 1993).

2.2.2. Metal efflux by metal transport proteins

Metal detoxification mechanisms are also based on the prevention of metal accumulation in the cytosol. Two strategies are reported: (i) the extrusion of toxic metal ions out of the cell via plasma-membrane transporters, and (ii) the sequestration of metals into intracellular compartments (Chardonnens *et al.*, 1999; Williams *et al*., 2000; Clemens, 2001; Hall, 2002; Peiter *et al.*, 2007). In plant, most of the transporters are member of the ZIP (ZRT, IRT-like protein) (Guerinot, 2000), the HMAs (Heavy Metal ATPases) (Hall and Williams, 2003), the Nramp (Natural resistance-associated macrophage protein) and the CDF (Cation Diffusion Facilitator) families (Williams *et al.*, 2000). In fungi, similar mechanisms can function to avoid cellular damage caused by the excess of metals in the cytosol. Most of our knowledge on metal transporters in fungi comes from studies on the yeast *S. cerevisiae* (Eide, 1998; Eide, 2006; Eide, 2009; Rutherford and Bird, 2004) (Fig. 4). However, some information about other fungi is also available.

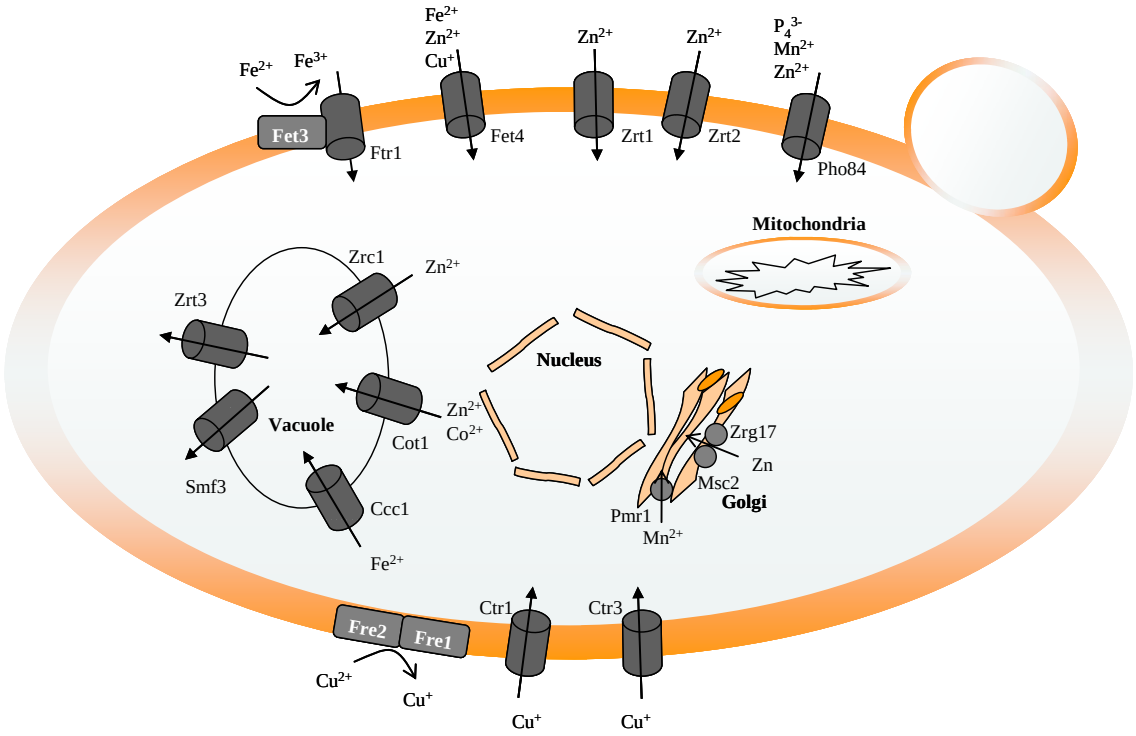

Figure 4. Metal ion transport in *S. cerevisiae* (Modified from Eide, 2006; Eide, 2009; Rutherford and Bird, 2004).

Most heavy metal resistance systems involve the efflux of toxic ions outside of the cells or, in eukaryotes, the sequestration in intracellular compartments. In bacteria, heavy metal resistance is mostly accomplished by exclusion systems (Nies, 2003). Different protein families include metal-exporting proteins. The P-type ATPases are a class of transporters identified in a wide range of organisms and are involved in the transport of essential and potentially toxic metals across cell membranes. Saitoh *et al.* (2009) divided the heavy-metal ATPases (HMAs) in fungi into three groups according to their function as transporter of copper ions to proteins or as cell-membrane copper-efflux pumps. Tolerance to Al of isolates of the mycorrhizal fungus *Pisolithus tinctorius* have been attributed to a metal exclusion mechanism (Egerton-Warburton and Griffin, 1995). In Zn-tolerant Suilloid strains, it was suggested the existence of a Zn exclusion mechanism (Colpaert *et al.*, 2005). Such a mechanism was also hypothesized to be involved in the protection of the host plant from toxic concentrations of heavy metals. In fact, the authors suggest that the accumulation of metal ions in the fungal vacuoles could decrease the metal transfer to the host plant.

Different studies considered the fungal vacuole as the main organelle for excess metal storage. In *S. cerevisiae*, the two CDF transporters *zrc1* and *cot1* are involved in Zn, Co and possibly Cd storage and detoxification (Gaither and Eide, 2001). These proteins are critical for heavy metal tolerance, indicating that the vacuole plays a key role in the resistance to toxic

metal concentrations (Eide, 2009). In the ectomycorrhizal fungus *P. involutus*, a vacuolar compartmentalization has been suggested as an essential mechanism for Cd detoxification (Blaudez *et al*., 2000). In an overview of the mechanisms evolved by *G. intraradices* to survive in metal contaminated soils, Ferrol *et al*. (2009) mentioned the important role of the metal transporters in the translocation of toxic metals into sub-cellular compartments, mainly vacuoles. Two Cu-transporters have been identified at the membrane vacuole of this mycorrhizal fungus: the ABC transporter GintABC1 (González-Guerrero *et al.*, 2007), and a P-type Cu-ATPase (Benabdellah *et al.*, 2007). The same group, was also able to isolate the first zinc transporter described in arbuscular mycorrhizal fungi (GintZnT1), which is a member of the CDF family. They reported that GintZnT1 has a role in Zn compartmentalization and may contribute in the tolerance mechanisms (González-Guerrero *et al.*, 2005). From a cDNA library constructed by using mRNA from Zn-treated *O. maius* mycelia, a protein with high homology with the *Schizosaccharomyces pombe* vacuolar ABC transporter (*hmt1*) was identified (Vallino *et al*., 2005). This protein is responsible for the transfer of the Cd-phytochelatin complexes from the cytosol to the vacuole (Ortiz *et al*., 1995) but the role in *O. maius* remains to be elucidated.

2.3. Antioxidant mechanisms

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. ROS are formed as a natural byproduct of the normal oxygen metabolism. However, ROS levels can increase in the presence of redox-active (Cu, Fe) or even redox-inactive metals (Zn, Cd) (Schutzendubel and Polle, 2002). In fungi, ROS are involved in cell differentiation, in mycorrhizal symbiosis, and in virulence and pathogenicity (Aguirre *et al.*, 2005; Baptista *et al*., 2007; Egan *et al*., 2007; Molina and Kahmann, 2007). Many diseases are related to oxidative damages as a result of an imbalance between radical-generating and radical-scavenging systems. Despite the presence of various production sites throughout the cell, ROS are mainly generated in mitochondria upon over-reduction of the electron transport chain and through various oxidases (Turrens, 2003; Aguirre *et al.*, 2005; Takemoto *et al.*, 2007). To protect against oxidative damages, antioxidant defense mechanisms are present to maintain ROS at basal level and to repair cellular damage (Fridovich, 1998). These mechanisms include both non-enzymatic antioxidants such as glutathione (GSH) and vitamins C (ascorbate), E (α-tocopherol) and B6 (Evans and Halliwell, 2001), as well as enzymatic antioxidant such as superoxide dismutases, catalases, peroxidases and glutaredoxins (Yu, 1994; Neill *et al*., 2002).

2.3.1. Non-enzymatic antioxidants

2.3.1.1.Glutathione (GSH)

Glutathione (gamma-L-glutamyl-L-cysteinyl-glycine: GSH) is one of the most abundant low molecular weight thiols. It is present in most eukaryotic cells and also in many prokaryotes (Penninckx and Elskens, 1993), and it is thought to play a vital role in buffering the cell against ROS (Stephen and Jamieson, 1996). In fungi, GSH is involved in the transport of amino acids into the cells and the vacuoles, the protection against oxidative stress, the detoxification of xenobiotics and heavy metals and it also takes part in the regulation of cell morphology and life cycle (Thomas *et al.*, 1991). Glutathione functions as an electron donor for glutathione peroxidase (GPx) that reduces hydrogen peroxide (H_2O_2) to H_2O and contributes to ROS degradation (Meister and Anderson, 1983). In metal tolerance mechanism, GSH can operate with different ways such as metal chelation and sequestration, protection against metal-induced oxidation, and binding to reactive sulphhydryl groups of proteins (glutathionylation). Synthesis of GSH involves two sequential steps catalyzed by distinct enzymes. GSH could reduce a number of oxidants, through the enzyme GPx, or via non-enzymatic way, giving rise to its oxidized form, glutathiol or oxidized glutathione (GSSG), by linking two cysteine residues. GSSG is recycled and reduced to GSH by an NADPH-dependant reaction catalyzed by glutathione reductase (GR) (Li *et al.*, 2009). In the filamentous fungus *Penicillium chrysogenum*, GPx, GR and glutathione-producing activities increase in response to oxidative stress (Emri *et al.*, 1997, 1999). Similarly, in *Aspergillus niger*, the addition of H_2O_2 leads to an increase in the intracellular pool of GSH (Li *et al.*, 2008).

2.3.1.2.Vitamins

Vitamins, such as vitamin C (ascorbate), E and B6, have been also considered as efficient antioxidant in cells. L-ascorbic acid, which is an efficient free radical scavenger with important metabolic functions, is produced by plants (Wheeler *et al*., 1998) and mammals (Meister *et al.*, 1994). However, it appears that fungi do not normally synthesize ascorbate and the species investigated contain D-Erythroascorbate (D-EAA), a C_5 ascorbate analogue. The presence of D-EAA instead of L-ascorbate has been observed in Ascomycete (Nick *et al*., 1986), Basidiomycetes (Okamura, 1994) and Zygomycetes (Baroja-Mazo *et al.*, 2005). In the zygomycete fungus *Phycomyces blakesleeanus*, the two compounds D-Erythroascorbate (D-EAA) and D-erythroascorbate glucoside (D-EAAG) can act as antioxidants (Baroja-Mazo *et al.*, 2005). Vitamin E is believed to be one of the major components in the protection against ROS and lipid peroxidation (Akiyama, 1999). Vitamin E acts as a lipid-based radical chain

breaking molecule with scavenging capacity for free radicals such lipid peroxyl, alkoxyl and hydroxyl radicals (Mikai *et al.*, 1988). Recently, in the arbuscular mycorrhizal fungus *G. intraradices*, up-regulation by superoxide and Cu of a gene encoding a protein involved in vitamin B6 suggests its potential role in the protection against oxidative stress (Benabdellah *et al.*, unpublished data).

2.3.2. Enzymatic antioxidants

2.3.2.1.Superoxide dismutase (SOD)

Superoxide dismutase (SOD) plays a major role in the antioxidant defense by catalyzing the dismutation of O_2^- radicals to H_2O_2 and O_2. H_2O_2 is subsequently removed by catalases, Glutathione peroxidase (GPx) and/or peroxiredoxins (PRXs). On the basis of the metal at the active site, SODs are classified into iron SOD (Fe-SOD), manganese SOD (Mn-SOD), Copper-Zinc SOD (Cu/Zn-SOD), and Nickel SOD (Ni-SOD) (Miller, 2004). *S. cerevisiae* possesses two SODs: the Cu/Zn-SOD (product of the *sod1* gene) and the Mn-SOD (product of *sod2* gene) (Fridovich, 1995). Cu/Zn-SOD was initially reported to be localized into the cytosol, but it has also been found in the mitochondrial intermembrane space (Sturtz *et al*., 2001; Nedeva *et al*., 2004), while, the Mn-SOD is located in the mitochondria matrix. Generally, the Cu/Zn-SOD has a major role in the first defense line against ROS, while the Mn-SOD is only involved in the protection mechanisms within mitochondria (Li *et al*., 2009). The two previously mentioned SODs have been also described in other fungi. In *Penicillium chrysogenum*, the presence of menadione in the medium induces an increase of both the Cu/Zn-SOD and Mn-SOD activities (Emri *et al*., 1999). Similarly, in *Aspergillus niger*, the response to oxidative stress caused by the addition of exogeneous H_2O_2, menadione (Kreiner *et al.*, 2002), and cadmium (Todorova *et al*, 2008) correlated with an increase of the SOD activity. Guelfi and colleagues (2003) reported an increase of the SOD activity in *A. nidulans* in response to Cd. To search for genes that might be involved in metal tolerance in the ectomycorrhizal fungus *P. involutus*, Jacob *et al*. (2001) isolated a full-length cDNA encoding a SOD enzyme, and observed that this gene rescue the transformed *E. coli*-SOD null mutant from oxidative stress generated by paraquat and cadmium. Recently, for the ericoid fungus *Oidiodendron maius* the first Cu/ZnSOD-targeted disruption in a mycorrhizal fungus was achieved via homologous recombination. The disruption of the *OmSOD1* gene resulted in the increased sensitivity to Cd, Zn and menadione and an increase of ROS production. In addition, it was demonstrated the role of *OmSOD1* as an important factor in the mycorrhizal relationship between *O. maius* and its host plant (Abbà *et al.*, 2009).

2.3.2.2.Catalases (CAT)

Catalases catalyse the decomposition of H_2O_2 to H_2O and O_2, thus reducing the intracellular hydrogen peroxide levels (Chelikani *et al.*, 2004). *S. cerevisiae* has two catalases (Traczyk *et al.* 1985; Skoneczny and Rytka, 2000), a peroxisomal catalase (catalase A: CTA1) and a cytosolic catalase (catalase T: CTT1). The double *cta1/ctt1* mutant is hypersensitive to peroxide suggesting that both catalases play an important role in the tolerance to oxidative stress (Izawa *et al.*, 1996). In filamentous fungi, more than one catalase gene exists and they are differentially regulated. In *A. nidulans*, two catalases were identified, *catA* and *catB* (Navarro *et al.*, 1996; Kawasaki *et al*., 1997; Navarro and Aguirre, 1998). Both genes could be induced by a variety of stresses: *catA* is increased in the presence of oxidative, osmotic, and nitrogen/carbon starvation stresses, while, *catB* is induced mainly by H_2O_2, paraquat and heat shock. Moreover, a high concentration of $CdCl_2$ induced a small increase in total Cat activity in *A. nidulans* (Guelfi *et al*., 2003). Homologous of *catA* and *catB* have been identified in other *Aspergillus* species such as *A. oryzae* (Hisada *et al.*, 2005) and *A. fumigatus* (Takasuka *et al.*, 1999). In *Neurospora crassa*, two catalases have been identified CAT1 and CAT3 (Schliebs *et al.*, 2006). Both catalases could be induced by H_2O_2, paraquat and cadmium. In the fungal pathogen *Cryptococcus neoformans*, four catalases were identified (CAT1, CAT2, CAT3 and CAT4). These catalases have been demonstrated to be involved in the antioxidant defense system. Giles and colleagues (2006) hypothesized that the catalases might contribute to resistance against oxidative stress via one of two models: (i) the activities of individual catalases might contribute to resistance against oxidative stress independently, or (ii) the catalase gene family members might function cooperatively.

2.3.2.3.Peroxidases

Fungal peroxidases have been classified into two groups: glutathione peroxidases (GPx), which employ GSH, and peroxiredoxins (PRXs), which employ thioredoxins (TRXs) as reductants.

2.3.2.3.1. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) is a type of peroxidase, whose electron donor is GSH. Unlike catalases, GPx could reduce also organic peroxides in addition to H_2O_2. In *S. cerevisiae*, three GPx were identified and named *GPX1*, *GPX2* and *GPX3* (Inoue *et al.*, 1999). In this work, they found that the gene expression was induced by oxidative stress only in *GPX2*. In fungi, several authors have reported GPx-like activities. In *P. chrysogenum*, GPx activity is induced

by superoxide and H_2O_2, (Emri *et al.*, 1997; Emri *et al.*, 1999), while in *A. niger* the GPx is thought to play a minor role in in destroying H_2O_2 (Bai *et al*., 2003).

2.3.2.3.2. Peroxiredoxin (PRX)

Peroxiredoxins (PRXs) are a family of thiol-specific peroxidases, and they are present in all kingdoms of living organisms. The peroxidase and peroxynitrite reductase activities of PRXs depend on the thiol-disulfite transition of cysteines supported by electron donors such as thioredoxin (TRx) and cyclophilin. PRXs are divided into three classes: typical 2-Cys PRXs; atypical 2-Cys PRXs; and 1-Cys PRXs (Wood *et al.*, 2003). Typical 2-Cys PRXs are homodimeric and require two conserved cysteine residues, with an intersubunit disulphide formed between them during the catalytic cycle. Atypical 2-Cys also require two cysteine residues for catalysis, although only the N-terminal one is conserved, and an intramolecular disulphide between both residues is formed during substrate reduction. 1-Cys PRXs are monomeric and employ a single active site cysteine. *S. cerevisiae* cells have five PRXs: Tsa1, Tsa2 and Ahp1 are cytosolic, Prx1 and Dot5 are mitochondrial (Park *et al*., 2000). In yeast lacking all five PRXs, cells are more susceptible to oxidative and nitrosative stress and the expression of the glutathione peroxidase and glutathione reductase was induced (Wong *et al*., 2004). In the fungal pathogen *C. albicans*, a homologue of the yeast Tsa1 have been identified and reported to confer resistance towards oxidative stress (Urban *et al.*, 2005).

2.3.2.4.Glutaredoxin (GRX)

Glutaredoxins (GRXs) are thiol-disulfide oxidoreductases involved in the catalysis of thiol-disulfide interchange reactions. GRXs reduce protein-glutathione mixed disulfides to protein thiols in the presence of GSH (Holmgren, 1976). The reaction involves reduced GSH as hydrogen donor, with the participation of cysteine residues of the enzyme active site (Holmgren, 1989; Holmgren and Aslund, 1995). GRXs can be subdivided into two subfamilies according to their active sites: the dithiol GRXs, with CPY/FC motif, and the monothiol GRXs, with the CGFS motif. Both subfamilies share a thioredoxin-fold structure. Most fungi have both subfamilies with different subcellular locations (Herrero *et al.*, 2006). The dithiol GRXs subfamily seem to play an important role in the protection against oxidative stresses in bacteria (Carmel-Harel and Storz, 2000), filamentous fungi (Benabdellah *et al.*, 2009), yeast (Luikenhuis *et al*., 1998; Lewinska and Bartosza, 2008), plant (Rouhier *et al.*, 20004) and mammalian (Lillig *et al.*, 2005) cells. The monothiol GRXs is also conserved across organisms, but a unifying function for this subfamily has not ascertained yet. *S. cerevisiae* possesses seven GRXs (GRX1-7). GRX1 and GRX2 belong to the dithiol

subfamily (Luikenhuis *et al.*, 1998), and GRX3 to GRX7 are members of the monothiol subfamily (Rodríguez-Manzaneque *et al*., 1999; Lee *et al*., 2007; Mesecke *et al.*, 2008). GRX1 confers protection against superoxide anion and hydroperoxides, while GRX2 is specialized in the protection against hydroperoxides (Luikenhuis *et al.*, 1998). Despite the important role of the GRX family to maintain the redox homeostasis of aerobic organisms, little is known about this gene family in fungi other than yeasts. Recently, Benabdellah and colleagues (2009) isolated the first gene (GintGRX1) belonging to the dithiol GRXs subfamily in *G. intraradices*, and concluded that GintGRX1 has a role in protecting the fungus against oxidative damage induced directly by superoxide anion or indirectly by copper.

3. Aim of the thesis

3.1. *O. maius* strain Zn: a good model to study the molecular basis of the oxidative stress tolerance mechanisms

Ericoid fungi are a diverse group of soil fungi that establish a distinctive type of mycorrhizal association with ericaceous plants (Bonfante and Gianinazzi-Pearson, 1979; Perotto *et al.*, 2002). These fungi profusely colonize the fine hair roots typical of ericaceous plants and, due to their saprotrophic capabilities, play an important role in mineral cycling and plant nutrition (Read, 1996). Thanks to their mycorrhizal status, host plants can grow on low-mineral, acidic, organic soils high in toxic metal ions, where they can become dominant species (Smith and Read, 1997).

The mechanisms that protect the host plants from heavy metal toxicity are, however, poorly understood (Perotto and Martino, 2001). It is also unclear how the ericoid fungi themselves survive and tolerate toxic concentrations of heavy metals, and whether they rely on cellular and molecular mechanisms already described for other organisms (Vallino *et al*., 2005).

The fungal isolate investigated in this study is the ericoid fungus *Oidiodendron maius* (Barron, 1962) strain Zn, deposited in the MUT collection at the Department of Plant Biology, University of Turin (MUT 1381). This strain was isolated from the Niepolomice Forest (25 km N.E. of Krakow, Poland) from the roots of *Vaccinium myrtillus* growing in experimental plots treated with 5000 t km^{-2} of dusts containing high concentrations of zinc, cadmium and aluminium derived from industrial electro-filters (Martino *et al*., 2000b).

Among mycorrhizal fungi, *O. maius* is a unique and powerful model system because it can be easily grown *in vitro*, where it reproduces asexually by forming conidia with a single haploid nucleus that can germinate and produce a homokaryotic mycelium.

O. maius Zn has been demonstrated to tolerate concentrations of Zn that cause a complete growth inhibition of isolates coming from unpolluted soils (Martino *et al*., 2000b). A number of studied were conducted to elucidate *O. maius* Zn tolerance mechanisms. Among the first suggested mechanisms there was the production of mucilage and extracellular pigments (Martino *et al*., 2000a). The influence of zinc ions on the secretion and activity of polygalacturonase (PG), an extracellular enzyme that hydrolyses the pectin component of the plant cell walls, was investigated because of its significance during saprotrophic growth. Addition of increasing concentrations of Zn and Cd ions to the purified enzymes resulted in an increase of PG activity (Martino *et al*., 2000a). The response of PG to heavy metal ions might have been a pre-adaptive factor for the colonisation of polluted soils by *O. maius*. In fact oligalacturonans may function as better metal chelators than larger polymers, thus protecting the fungus during saprotrophic growth. Moreover zinc ions induced in *O. maius* a general change in the array of secreted proteins, with a shift towards the production of more basic, low molecular weight polypeptides. Among them were hydrolytic enzymes (nuclease, proteinase, lysozyme) and two superoxide dismutase isoforms (Martino *et al*., 2002), known to play a pivotal role in heavy metal response in plants, animals and microorganisms. Further investigations focussed on the interactions between insoluble metal species and extracellular fungal metabolites (Martino *et al*., 2003) (Fig. 5). *O. maius* Zn was demonstrated to mobilize insoluble inorganic zinc compounds to a lower extent when compared with fungi coming from unpolluted soils. This result may reflect a specific strategy of *O. maius* Zn to maintain homeostasis of essential metals under polluted soil conditions. Induction of organic acids (malate and citrate) by the metal compounds was at least in part responsible for metal solubilization. *O. maius* Zn was also demonstrated to have some weathering capacities, it can modify asbestos fibres *in vitro* by removing the ROS- generating surface iron (Martino *et al*., 2004) (Fig. 5). The genetic basis of this fungal strain tolerance to high zinc concentrations were also investigated by using an untargeted approach. From a cDNA library constructed by using mRNA from Zn-treated *O. maius* mycelia, 444 clones were randomly selected and 318 were sequenced (Vallino *et al*., 2005). Among the identified proteins were a Cu/Zn superoxide dismutase (SOD), an ABC transporter and several antioxidant enzymes. The Cu/Zn superoxide dismutase isolated from the cDNA library corresponded to that previously identified through protein microsequencing (Martino *et al.*, 2002; Vallino *et al.*, 2009). Functional complementation assays showed that the gene confers increased tolerance to zinc and copper stress to a Cu,ZnSOD-defective yeast mutant. Monitoring of transcript and protein levels following zinc stress suggests that OmSOD1 expression is controlled at the transcriptional level. The OmSod1 protein was found both in the cell extract and in the growth

medium of viable fungal cultures. This was the first characterization of an extracellular Cu,ZnSOD in a mycorrhizal fungus. In nature, the presence of OmSod1 in the extracellular environment may also extend the protective role of this enzyme to the plant symbiont (Vallino *et al*., 2009). Moreover, Vallino *et al.* (2011) demonstrated that *O. maius* Zn, showed a significantly higher polymorphism in the Cu,ZnSOD promoter region, when compared with isolates coming from a naturally metal enriched area, suggesting that environmental stress may influence the rate of mutations in specific regions of the Sod1 locus. Genetic trasformation of *O. maius* by protoplasts and *Agrobacterium*-mediated transformation (AMT) have been developed and it has also been used to express GFP constructs in order to follow mycorrhizal colonization (Martino *et al*. 2007) (Fig. 5). For further investigations a comparative proteomics approach was used (Chiapello *et al*., unpublished) to elucidate common and specific responses of *O. maius* Zn to zinc and cadmium. Significant qualitative and quantitative changes in the fungus protein profiles were highlighted in the different conditions tested. Both metal ions strongly induced proteins involved in ubiquitination. Cadmium exposure selectively induced components of the translation machinery and molecular chaperons of the Hsp90 family, as well as some antioxidant proteins (e.g. thioredoxins). Zinc treatment again specifically induced a Cu/Zn SOD. The results indicate common but also metal specific pathways activation upon cadmium and zinc exposure, especially in the anti-oxidative stress response.

Genes that may be responsible of *O. maius* survival and growth when exposed to cadmium have been recently isolated from a complementation library in a yeast sensitive mutant (Abbà *et al*., unpublished).

Finally, genomic and transcriptomic tools are recently becoming available for *O. maius* strain Zn by 454 pyrosequencing, yielding a large database of gene sequences for this organism, which is now also included in an international sequencing project of the *Joint Genome Institute* (JGI) named Exploring the Genome Diversity of Mycorrhizal Fungi to Understand the Evolution and Functioning of Symbiosis in Woody Shrubs and Trees.

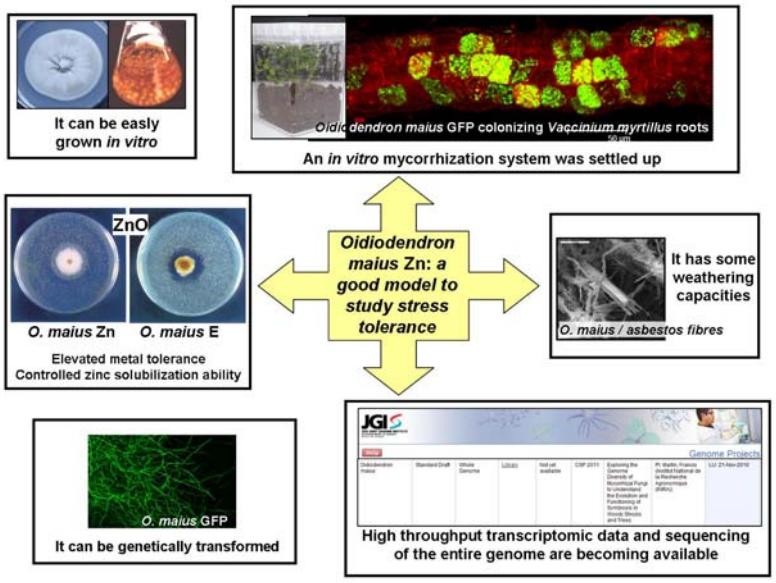

Figure 5. Schematic representation summarizing some of the results obtained for *O. maius* Zn in the studies previously performed.

The specific aim of my thesis was to identify other genes potentially involved in the elevated metal tolerance observed for *O. maius* strain Zn using three different approaches:

(i) The first approach was based on the application of fungal genetic transformation techniques and gene disruption. This approach led to the generation of *O. maius* (SOD1)-null mutants and this was the first example of a gene-targeted disruption via homologous recombination in a mycorrhizal fungus.

(ii) The second approach was based on the screening of *O. maius* cDNA library transformed in the yeast mutant *zrc1*, which is devoid of a zinc vacuolar transporter. This screen resulted in the isolation and identification of two zinc transporters which were further characterized.

(iii) The third approach consisted in the screening of a collection of *O. maius* Zn random-mutants for sensitivity to Zn, Cd and Menadione, and in the identification of the mutated genes. Three genes were identified through the TAIL-PCR technique and one was characterized more in detail.

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Internet resources

Periodic table of the elements

http://blogs.4j.lane.edu/taylor_k/2010/06/01/metals-this-week-in-science-june-1-june-4/

Chapter 2

SOD1-targeted gene disruption in the ericoid mycorrhizal fungus *Oidiodendron maius* reduces conidiation and the capacity for mycorrhization

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Abstract

The genome sequences of mycorrhizal fungi will provide new opportunities for studying the biology and the evolution underlying this symbiotic lifestyle. The generation of null mutants at the wild-type loci is one of the best methods for gene function assignment in the post-genomic era. To our knowledge, the generation of SOD1-null mutants in the ericoid mycorrhizal fungus *Oidiodendron maius* is the first example of a gene targeted disruption via homologous recombination in a mycorrhizal fungus. The disruption of *OmSOD1* by *Agrobacterium*-mediated transformation resulted in the presence of oxidative stress markers, even in the absence of external superimposed stresses and an increased sensitivity to ROS-generating substances, especially to menadione. A reduction in conidiation and in the percentage of mycorrhization of *Vaccinium myrtillus* roots was also observed. The latter findings establish the pivotal role of SOD1 as an important factor in the relationship between *O. maius* and its symbiotic partner. The lack of this ROS-scavenger is suggested to cause an imbalance in the redox homeostasis during host colonization and an alteration in the delicate dialogue between the fungus and its host plant.

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1. Introduction

The complex communication that must occur during mycorrhizal symbiosis between the fungus and the plant is likely to be explained in the coming years through comparative genomics, because a large amount of information from the genome sequences of fungal saprotrophs, pathogens and symbionts is now available (Martin *et al*., 2007). The release of the complete *Laccaria bicolor* genome (Martin *et al*., 2008) and the ongoing sequencing of *Tuber melanosporum* (http://mycor.nancy.inra.fr/IMGC/TuberGenome/) and *Glomus intraradices* (http://www.jgi.doe.gov/sequencing/statusreporter/psr.php?projectid=16063) genomes are going to provide further opportunities for studying the biology and the evolution underlying this symbiotic lifestyle. Gene function assignment then becomes the new challenge and depends on the development of high-throughput methodologies to test gene function *in vivo* or *in vitro*. One such method is the generation of null mutants via homologous recombination at the wild-type loci by using inactivation cassettes.

The first evidence for genetic transformation of a mycorrhizal fungus (Barrett *et al*., 1990) was the successful transformation of the basidiomycete *L. laccata* using protoplast-PEG-based transformation. Since then, other mycorrhizal species were stably transformed: several ectomycorrhizal fungi, all basidiomycetes, such as *Hebeloma cylindrosporum* (Combier *et al*., 2003; Pardo *et al*., 2002; Marmeisse *et al*., 1992), *Paxillus involutus* (Pardo *et al*., 2002; Bills *et al*., 1995), *Laccaria bicolor* (Kemppainen *et al*., 2005; Bills *et al*., 1999), *Suillus bovinus* (Pardo *et al*., 2002; Hanif *et al*., 2002) and *Pisolithus tinctorius* (Rodriguez-Tovar *et al*., 2005), and one ericoid mycorrhizal (ERM) fungus, the ascomycete *Oidiodendron maius* strain Zn (Martino *et al*., 2007). The random integration of a selectable marker into the host genome can lead to the creation of large collections of random mutants which are then screened for a phenotype of interest. By contrast, homologous recombination has not been achieved yet for mycorrhizal fungi.

In this work, we have obtained the first targeted gene inactivation in a mycorrhizal fungus. *Oidiodendron maius* is one of the most widely investigated ERM fungi. The symbiotic association between ericaceous plant roots and their endophytic fungi is known to facilitate the uptake by plants of organic nitrogen from recalcitrant organic compounds. Beside the nutritional benefits, ERM fungi also play a crucial role in the protection of their host plants against heavy metal toxicity through their ability of complexing and compartmentalizing excess toxic elements (Turnau *et al*., 2007; Sharples *et al*., 2000). In particular, *O. maius* strain Zn, which was isolated from industrial dusts-contaminated plots, has been demonstrated

to tolerate concentrations of zinc that cause a complete growth inhibition of isolates coming from unpolluted soils (Martino *et al*., 2000).

O. maius can be easily grown *in vitro*, where it reproduces asexually by forming conidia with just a single haploid nucleus. This characteristic makes this fungus a good candidate to study mutants, because uninucleated spores can germinate and produce a homokaryotic mycelium with all the nuclei carrying the mutation. Moreover, the haploid monokaryotic status implies that the modifications of the wild-type phenotype, if detectable, can be observed even when mutations are recessive.

Agrobacterium-mediated transformation (AMT) was recently used for random insertional mutagenesis to generate mutants of this fungus (Martino *et al*., 2007). In this work, we have used the same transformation method to specifically delete the single copy gene *OmSOD1*, the copper and zinc containing superoxide dismutase - EC 1.15.1.1. of *O. maius* Zn (Vallino *et al*., 2009).

This gene has a two-fold importance, because it has been recently proposed to play a role in the differentiation processes associated with fungal symbiosis (Tanaka *et al*., 2006) and it is a key enzyme in the detoxification of the cell from reactive oxygen species (ROS) as described in many other fungi, such as *Saccharomyces cerevisiae* (Gralla and Valentine, 1991), *Aspergillus nidulans* (Guelfi *et al*., 2003), *A. niger* (Todorova *et al*., 2007), *P. involutus* (Jacob *et al*., 2001) and *Schizosaccharomyces pombe* (Tarhan *et al*., 2007). ROS are by-products of normal metabolism, but their level dramatically increases in the presence of redox-active metals such as Fe and Cu (Schutzendubel and Polle, 2002) or superoxide ion-generators such as the redox cycling agent menadione (Emri *et al*., 1999). Interestingly, the occurrence of activated oxygen and symptoms of oxidative injury have also been observed in organisms exposed to non-redox-active metals like Zn or Cd (Schutzendubel and Polle, 2002). In most cases, exposure to these metals initially resulted in a severe depletion of free-radical scavengers, such as thiol-containing compounds, and in increasing ROS levels (Dietz *et al*., 1999).

Given the functional dualism of the SOD1 protein, we took advantage of our experimental organism which is both a mycorrhizal and a zinc-tolerant fungus to assess whether a) the lack of a ROS scavenger can alter its ability to establish a mycorrhizal symbiosis with the roots of its host plant; b) the deletion of the *SOD1* gene can influence the fungal response to the oxidative stress caused directly by a ROS generating agent, such as menadione, and indirectly by zinc and cadmium.

2. Materials and Methods

2.1. Fungal isolate and growth media

The fungal isolate investigated in this study is *Oidiodendron maius* strain Zn, deposited in the MUT collection at the Department of Plant Biology, University of Turin (CLM1381.98). This strain was isolated from the Niepolomice Forest (25 km N.E. of Krakow, Poland) from the roots of *Vaccinium myrtillus* growing in experimental plots treated with 5000 t km^{-2} of dusts containing high concentrations of zinc (Zn), cadmium (Cd) and aluminium derived from industrial electro-filters. The characteristics of the site and the identification of this fungal isolate are described in more detail by Martino *et al.* (2000).

Fungi were grown in Czapek mineral medium supplemented with 2% (w/v) glucose (Sigma). The mineral medium contained $NaNO_3$ (3 g l^{-1}), $K_2HPO_4.3H_2O$ (1.31 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}), KCl (0.5 g l^{-1}), $FeSO_4.7H_2O$ (0.01 g l^{-1}). When required, the medium was supplemented with either Zn^{2+} (as $ZnSO_4.7H_2O$, Fluka, 99% purity), Cd^{2+} (as $3CdSO_4.8H_2O$, Sigma, 98% purity) or menadione sodium bisulfite (95%, Sigma). The pH of solid media was adjusted to 4 using HCl 1M, while liquid media were buffered at the same pH using 20 mM MES (2-[N-morpholino] ethane sulphonic acid) before autoclaving.

2.2. Construction of the *OmSOD1* disruption cassette

The *OmSOD1* disruption cassette was designed as a gene replacement vector for the removal of a 621 bp fragment of the *OmSOD1* gene (GenBank accession number EU386164; Vallino *et al*., 2009), from 136 bp upstream to 485 bp downstream of the start codon (Fig. 1 A).

The *OmSOD1* disruption cassette carried the 786 bp-5' *SOD1* flanking region (from 135 bp to 921 bp upstream of the start codon) and the 820 bp-3' *SOD1* flanking region (from 486 bp to 1324 bp downstream of the start codon) amplified from the genomic *O. maius* Zn DNA, separated by the hygromycin resistance expression cassette containing the *A. 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).

Four PCR reactions were set up: three primary reactions to generate the three parts of the disruption cassette and a secondary reaction intended to fuse the three fragments into a single 5313 bp-long amplicon according to the protocol described by Kuwayama *et al*. (2002). The three primary 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 5x 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 1

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 1 cycle.

The 5'- and 3'- flanking regions of *OmSOD1* were amplified with primers 1/2 and 3/4 respectively (Table 1). The marker cassette DNA fragment was amplified with primer 5, complementary to primer 2, and primer 6, complementary to primer 3 (Table1). During the fusion PCR the 5'- and 3'- flanking regions were joined to the hygromycin cassette and the final PCR product was amplified with the *HindIII*–tailed primers 1 and 4. The fusion PCR reaction was carried out using 30 ng of the purified 5'- and 3'- PCR products and 90 ng of the purified hygromycin cassette following the same protocol previously described for the amplification of the three separate parts. Construction of the disruption cassette was confirmed by DNA sequencing.

Table 1. Primers for generating the *OmSOD1* disruption cassette by fusion PCR

Primer	Sequences
1[a]	5'-GGACTTGGAGCAGACGGTGC-3'
2[b]	5'-CTCCATGTTGGTAGTTGTGACAGGACAGGGATGTGCGTGCCTGGAGTTTTGCC-3'
3[b]	5'-CAAGCCTACAGGACACACATTCATCGACCCCCCAGAGTGTTATTGGCGTAAGTCG-3'
4[a]	5'-CGAGCACCTGGCTTAGAGGAGGC-3'
5	5'-GGCAAAACTCCAGGCACGCACATCCCTGTCCTGTCACAACTACCAACATGGAG-3'
6	5'-CGACTTACGCCAATAACACTCTGGGGGTCGATGAATGTGTGTCCTGTAGGCTTG-3'

[a] Primers 1 and 4 are complementary to the *OmSOD1* gene.
[b] Primers 2 and 3 (and their reverse complementaries 5 and 6) are chimerical primers: the underlined part of each primer is complementary to the *OmSOD1* gene, while the other part is complementary to the region of the *hph* gene which is going to be fused with the *OmSOD1* gene.

2.3. *Agrobacterium*-mediated transformation

To create the pΔ*SOD1* gene disruption construct, the *HindIII*-tailed *OmSOD1* disruption cassette was cloned into the *HindIII*-digested pCAMBIA0380 (CAMBIA) between the T-DNA borders. The vector was introduced into *A. tumefaciens* LBA1100, which carries the virulence (*vir)* genes essential for T-DNA transfer. This *A. tumefaciens* strain was used to transform the ungerminated conidia of *O. maius* Zn according to the protocol described by Martino *et al*. (2007). The only modification to this protocol was that the pH of the induction medium was set at 5, instead of 6. A pilot transformation experiment demonstrated that the number of transformants at pH 5 is almost 20-fold higher than at pH 6 (data not shown).

The transformants were isolated and transferred into 24-wells plates with Czapek-dox agar (1%, w/v) supplemented with 100 µg hygromycin/ml.

2.4. PCR screening for SOD1-null mutants

SOD1-nullmutants were primarily identified by PCR. A small portion of each fungal colony was collected, boiled for 15 min in 20 µl Tris-HCl 10mM pH 8.2 and vortexed for 1 min. Then, 2 µl were directly use for PCR amplification without any other purification. PCR

amplification was carried out in a final volume of 20 µl using REDTaq DNA Polymerase (Sigma) following the manufacturer's protocol. We used two sets of primers: set 1, 5'-CGCCACCACAGCTCTAGAGT-3' and 5'-CACGGATAAGCTGGTCAAGC-3'; set 2, 5'-ATGCCTGAACTCACCGCGAC-3' and 5'-GCAGTTCGGTTTCAGGCAGG-3'. The first set of primers was designed to amplify the DNA fragment to be replaced by the *hph* gene and removed from the disruptants. The second was designed to amplify the *hph* gene. If the fungus was a nonhomologous transformant, then an amplified product would be obtained with both sets of primers. If the transformant was a SOD1-null mutant, then an amplified product would be obtained only with the second primer set.

Gene disruption by homologous recombination was further checked by PCR using specific primers (set 3) designed on two *OmSOD1* regions external to the targeting construct: forward, 5'-ACGTGGTAGGGGCTAGCAGA-3' and reverse, 5'-TGGTCTTGATATGGACAGTTC-3'. Amplification with these primers was diagnostic of a targeted gene replacement resulting from accurate homologous recombination at each end of the construct. In the wild-type strain and in the nonhomologous transformants a 2310 bp-long fragment was expected, while a 5376 bp-long amplicon indicated the integration of the *OmSOD1* disruption cassette at the correct locus.

2.5. Southern Blot

The candidates for SOD1-null mutants were further confirmed by Southern hybridization analysis. Genomic DNA extracted from the PCR-selected SOD1-null mutants, the wild-type, and one ectopic transformant were digested with *PstI* and size-fractionated on a 1% (w/v) agarose gel. The separated restriction fragments were blotted onto nylon membrane following standard procedures (Sambrook and Russell, 2001). Hybridisation with a chemiluminescent detection system (ECL Direct DNA labelling and Detection System, Amersham) was performed according to the manufacturer's recommendations. An *OmSOD1*-specific probe and a *hph*-specific probe were used to confirm the absence of a functional *OmSOD1* gene in the SOD1-null mutants and a single copy integration of the disruption cassette in their genome.

2.6. Protein analysis

Three randomly chosen SOD1-null mutants with a single copy integration of the disruption cassette (D5_83, D5_125 and A4_57) and the wild-type were also tested by Western blot using a polyclonal rabbit anti-SOD1 primary antibody (Abcam plc, UK). Proteins were extracted from 30 day-old mycelia grown in 10 mM zinc-amended liquid medium using a

1mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM Na-phosphate buffer pH 7.5 and polyvinyl polypyrrolidone (PVPP) 8% (w/v). Following protein quantification with a Bradford assay (Sigma), 650 ng were loaded on 12.5% acrylamide separating gels. Proteins were then electrotransferred for 50min at 100V onto Hybond-ECL nitrocellulose paper (Amersham Pharmacia Biotech) and the membrane was hybridised overnight at 4°C with a 1:5000 dilution of the primary antibody. A goat anti-rabbit alkaline-phosphatase conjugated antibody (Sigma) was then diluted 1:5000 and used as secondary antibody. Analytical gels were Silver stained.

2.7. Determination of metal tolerance concentrations on agar medium

To determine the concentrations of zinc, cadmium and menadione that cause a drastic decrease in the radial growth as compared to the control, SOD1-null mutants and the wild-type were grown in 9-cm (diameter) Petri dishes containing the same mineral medium described above and different zinc (10-60 mM), cadmium (0.05–0.6 mM) and menadione (0.02-1 mM) concentrations. Radial growth was recorded after 30 days of growth at 25°C in the dark. At least three replicates were used for each experimental condition.

2.8. Determination of the capacity of conidiation

Conidia were harvested from three month-agar cultures of both wild-type and mutants by gently scraping cultures in 9 cm Petri dishes flooded with 1ml of sterilized distilled water.
Conidia were counted in a Bürker counting chamber (Marienfeld, Germany). Harvest from each fungus was performed in triplicate, and the mean and standard error of the total number of conidia per surface unit (cm^2) were reported.

2.9. NBT staining

Three hundred conidia of each fungus were inoculated in 12-wells plates containing 2 ml of liquid medium in each well. After 20 days of growth at 25°C in the dark, 10mM zinc, 0.05 mM cadmium and 0.02 mM menadione were added into the wells. After 3 hours of incubation, the medium was replaced by a 0.05% (w/v) NBT (nitroblue tetrazolium, Sigma) solution in 50mM sodium phosphate buffer, pH 7.5 (Munkres, 1990). After 3 hours of staining in the dark, the small colonies were placed on microscope slides and fixed with lactic acid for observation.
The colonies were examined and photographed with a Primo Star (Zeiss) compound microscope with a Moticam 2300 camera at x1000 magnification.

2.10. Mycorrhizal synthesis

Endomycorrhizae were synthesized aseptically using a nutrient agar culture system (Villarreal-Ruiz *et al*., 2004). Pot experiments with sterile substrates were avoided due to technical problems encountered in previous experiments (e.g. high risk of contaminations, difficult in determining fungal growth on the peat substrate). Plastic Petri plates (15 cm diameter, 2.5 cm height) containing autoclaved modified Ingestad's Solution (MISAG) (Ingestad, 1971), were prepared as follows: Solution B - NH_4NO_3 1.062 g l^{-1}, KNO_3 0.372 g l^{-1}, KH_2PO_4 0.286 g l^{-1}, K_2SO_4 0.222 g l^{-1}; Solution C (Stock Solution) - HNO_3 1.6 g l^{-1}, $Ca(NO_3)_2$ 14.3 g l^{-1}, $Mg(NO_3)_2$ 26.0 g l^{-1}, $Fe_2(SO_4)_3$ 2.50 g l^{-1}, $MnSO_4$ 0.55 g l^{-1}, H_3BO_3 0.57 g l^{-1}, $CuCl_2$ 0.032 g l^{-1}, $ZnSO_4$ 0.036 g l^{-1}, Na_2MoO_4 0.007 g l^{-1}. Solution B was mixed with Solution C (diluted 1:100) in equal amount. After the addition of 0.1% glucose, the pH was brought to 4.8 with KOH and the medium was finally supplemented with 1% agar. Axenic *V. myrtillus* seedlings were obtained from surface-sterilized (ethanol 70%, v/v, + Tween 20 0.2%: 3 min; 2x sterile water rinses, sodium hypochlorite 0.25%: 15 min, 3x sterile water rinses) bilberry seeds germinated on water - 1% agar Petri plates in the dark for 2 weeks and then transferred in the growth chamber for 1 month.

Four mycelium plugs (5 mm diameter) were removed from the leading edges of a 30 days-old fungal colony and placed in the bottom half part of a MISAG Petri plate at a distance of 2 cm from one another. Three germinated *Vaccinium myrtillus* seedlings were then transferred aseptically in the MISAG plates near the mycelium plugs (1 cm-distance between the plugs and the root system) (Fig. 1). Finally the plates were sealed and placed in a growth chamber (photoperiod 16 h; light 170 µmol m^{-2} s^{-1}; temperature 23°C/21°C day/night).

Two independent experiments were set up. In the first, three *Vaccinium* seedlings were used for each fungus and the root systems were observed after a 1.5-month incubation. In the second, three Petri dishes, each containing three seedlings (for a total of 9 replicates), were used for each fungus.

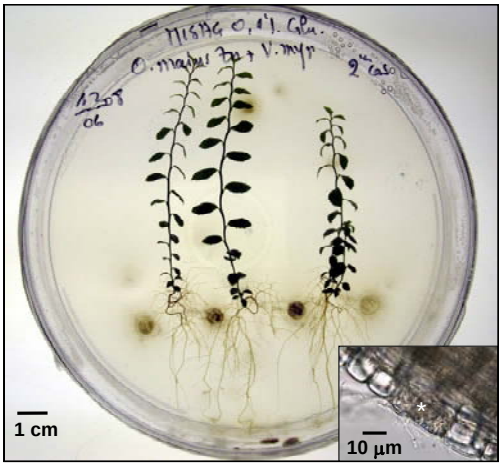

Figure 1. In vitro mycorrhization system involving *Vaccinium myrtillus* seedlings and *Oidiodendron maius*. After a month of pre-growth in 9 cm-Petri dishes, three axenic germinated *V. myrtillus* seedlings were aseptically transferred in a 15 cm-Petri plate near four mycelium plugs. The root systems were observed after 1.5 and 3 months of incubation in a growth chamber (photoperiod 16 h; light 170 µmol m^{-2} s^{-1}; temperature 23°C/21°C day/night). *O. maius* has formed a Cotton blue-stained coil (asterisk) in an epidermal cell (inset)

2.11. Plant growth parameters and degree of mycorrhization

Plant growth parameters and the percentage of mycorrhization were recorded after 3 months to determine differences in root colonization between *O. maius* Zn wild–type and the SOD1-null mutants. Length (cm) and fresh weight (g) of each stem and root system were measured and then each stem was inserted between two glass plates and scanned. Lucia G imaging software (version 4.82, Nikon) was used to evaluate the total leaf surface (cm^2) and the internodal distance (cm). Finally, stems were dried at 70°C for two days and weighed.

Each *Vaccinium* hair root system from the synthesis plates was cleared for 5 min with 10% (w/v) KOH in a water bath at 90°C, acidified with 0.1 M hydrochloric acid for 1 h, stained in 0.1 % (w/v) cotton blue for 15 min at 90°C, and destained overnight with lactic acid 80% (Fig. 4, inset). Whole roots were mounted in the destaining solution, observed using a Nikon Eclipse E400 optical microscope, and photographed. The magnified intersections method (Villarreal-Ruiz *et al.*, 2004) was adapted to quantify the percentage of infection of *Vaccinium* hairy roots. For each fungus, the root system of *Vaccinium* seedlings was examined under the microscope using the rectangle around the cross-hair as intersection area at x200 magnification. 100 intersections per seedling root system were scored. Counts were recorded as percentage of root colonized (RC) by the fungus using the formula: %RC = 100 x $\sum$ of coils counted for all the intersections /$\sum$ of epidermal cells counted for all the intersections.

2.12. Statistical analysis

The angular transformation was applied to the mycorrhization percentages. Statistical tests were all carried out through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $P < 0.05$.

3. Results

3.1. Gene disruption of *OmSOD1*

To determine if *OmSOD1* is essential in *O. maius* for resistance to heavy metals and for mycorrhization, a deletion construct that replaces almost all the coding sequence of the *OmSOD1* gene with the hygromycin resistance gene was designed and introduced into *O. maius* ungerminated conidia (Fig. 2 A). Hygromycin-resistant colonies were screened by PCR for homologous recombination. SOD1-null mutants were characterized by i) the absence of amplification with primer set 1, designed to amplify the DNA fragment replaced by the *OmSOD1* cassette; ii) a 5376 bp-long amplicon obtained with primer set 3, designed on the two *OmSOD1* regions external to the targeting construct. Thirteen putative homologous recombinants out of the 857 screened transformants were hence identified. Genomic DNA of these homologous recombinants, the wild-type strain, and the ectopic transformant B3_123 were digested with *PstI* for Southern hybridization analysis. *PstI* has no recognition sites either in the *OmSOD1* gene or in the *hph* gene covered by the *hph* probe. The absence of signal on all the PCR-selected SOD1-null mutants after hybridization with the *OmSOD1*-specific probe confirmed the *OmSOD1* gene disruption (data not shown). The same blot was also probed with a fragment of the hygromycin-resistance gene to verify the single integration of the construct at the *OmSOD1* locus (Fig. 2 B). Two copies of the expression cassette were integrated in two of the SOD1-null mutants (B5_75 and D3_98), while the other 11 showed a single integration.

The gene replacement (GR) frequency was defined as the number of homologous recombinant colonies divided by the total number of the analyzed hygromycin-resistant colonies. Based on the PCR and Southern analysis, the GR frequency obtained in this *Agrobacterium*-mediated transformation is 1.52% with *OmSOD1* homologous regions of 786 and 820 bp flanking the hygromycin-resistance cassette.

A4_57, D5_83 and D5_125 mutants were randomly chosen for further analyses among the 11 SOD1-null mutants with a single integration. After having confirmed the integration of the disruption cassette at the correct locus, we verified the loss of expression by Western blot analyses. The wild-type and the three selected mutants were grown in the same culture conditions (in liquid 10 mM zinc-amended medium for 30 days) that had been shown by Vallino and colleagues to induce the expression of the SOD1 protein (Vallino *et al*., 2009). Western blots showed a single band of the expected size in the wild type and no detectable SOD1 protein in the SOD1-null mutants (Fig. 2 C). RT-PCR of the RNA isolated from the same samples used for Western blot analyses revealed that in the mutants the *OmSOD1* gene was not transcribed in the mutant (data not shown).

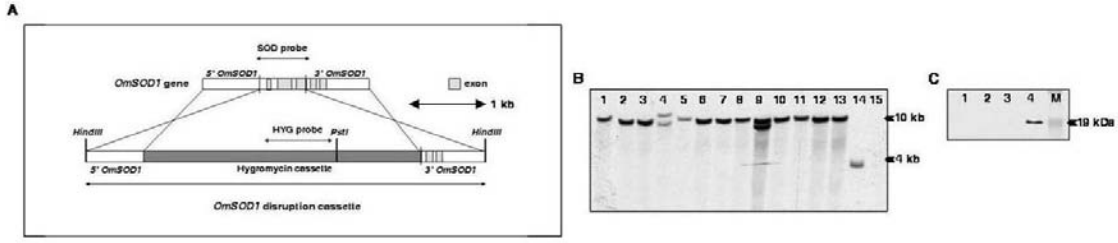

Figure 2. Disruption cassette and Southern Blot analysis A, Upper part, the wild-type OmSOD1 gene and the position of the five exons. Bottom part, the disruption cassette containing the 5'-flanking sequence and the remaining nonfunctional 3' part of the OmSOD1 gene. The hph gene and its promoter and terminator sequences are depicted as one unit within the disruption cassette to simplify the picture. The SOD1 and hph gene fragments used as probes in Southern blotting are indicated by black arrowed bars. The position of the PstI recognition site is also shown. B, Southern blot analysis of the recombination event. Genomic DNA was restricted with PstI and hybridised with hph probe. Lanes 1-13: PCR-selected SOD1-null mutants (C2_42, A4_57, D5_74, B5_75, C5_76, D5_83, D5_125, A4_95, D3_98, B4_97, C4_86, B1_84, C6_18); Lane 14: an ectopic transformant (B3_123); Lane 15: wild-type Oidiodendron maius Zn.The disruption cassette has integrated by homologous recombination in the genome of all the 13 transformants, but in B5_75 and D3_98 an ectopic integration also occurred. C, The Western Blot analysis of 650 ng of total protein from the selected SOD1-null mutants and the wild-type demonstrates a lack of SOD1 protein from SOD1-null mutants D5_83 (lane 1), D5_125 (lane 2) and A4_57 (lane 3) and a band at 19 kDa in the wild-type sample (lane 4). M, prestained protein molecular weight marker (Bio-Rad, Hercules, CA, U.S.A).

3.2. SOD1-null mutants have a significant growth defect on stressing media

The wild-type and the three mutants were grown on heavy metal- and menadione-amended media to evaluate their ability of tolerating increasing concentrations of these stress-inducing substances (Fig. 3). All the fungi showed a similar radial growth on the control media, while the addition of zinc, cadmium and menadione adversely affected the SOD1-null mutants more than the wild-type. Differences in the radial growth were clearly visible on zinc-, cadmium- and menadione-amended media starting from 30 mM, 0.3 mM and 0.5 mM, respectively. Especially on menadione, the mutants showed almost no growth at the highest concentrations applied, whereas the wild-type was still able to grow.

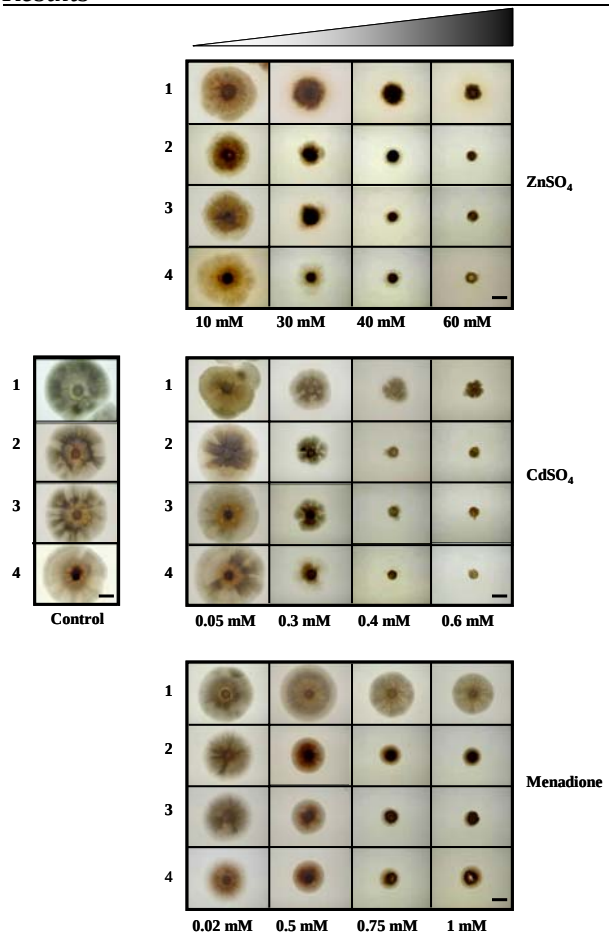

Figure 3. Determination of oxidative stress tolerance of SOD1-null mutants. The wild-type strain and the mutants were grown on agar medium for 30 days in Czapek-glucose amended or not with zinc, cadmium and menadione. The concentration of heavy metals and menadione rises from left to right. Concentrations of 10, 30, 40 and 60 mM zinc, 0.05, 0.3, 0.4 and 0.6 mM cadmium, 0.02, 0.5, 0.75 and 1 mM menadione were tested. All of the SOD1-null strains exhibited an increased sensitivity, especially to menadione, in respect to the wild-type. 1) *O. maius* Zn wild-type; 2) D5_83; 3) D5_125; A4_57. Bars = 1 cm

3.3. A reduced conidiation was observed in SOD1-null mutants

Based on one-way ANOVA, the number of conidia per surface unit produced by the SOD1-null mutants was significantly lower in respect to the wild-type: 1.05 ± 0.11 (figures are expressed in millions) conidia/cm^2 were counted for the wild-type, while 0.61 ± 0.08, 0.64 ± 0.06, 0.65 ± 0.03 for D5_125, D5_83 and A4_57, respectively. Thus, an average 1.5 fold-reduction in conidiation capacity was recorded in the mutants.

3.4. ROS production

Mycelial samples of the wild-type and the mutants grown both in control and in stress-inducing media were examined for their ability to generate superoxide anions by reduction of nitroblue tetrazolium (NBT) to dark-blue, water-insoluble formazan. Within 3 hours after NBT treatment, hyphae of the mutants grown in control medium were clearly stained with dark-blue formazan spots, while no deposits were found in the wild-type (Fig. 4). No significant differences were observed in the pattern and quantity of the deposits between the control and the stressed hyphae both in the mutants and in the wild-type (data not shown).

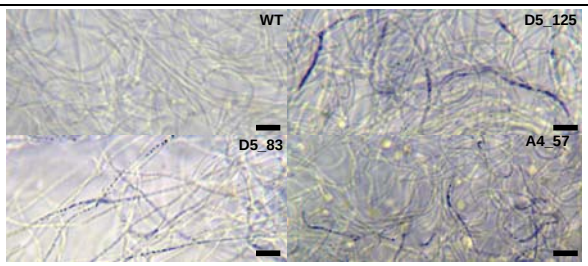

Figure 4. ROS production by *O. maius* wild-type and SOD1-null mutants in liquid control medium. Light microscope photographs of colony mycelia of *O. maius* Zn wild-type (WT) and SOD1 null mutants (D5_83, D5_125 and A4_57) grown on Czapek-glucose agar medium and stained with 0.05% (w/v) NBT solution for 3 h. Bars = 10 μm.

3.5. Plant growth parameters and degree of *in vitro* mycorrhization

We investigated whether the SOD1-null mutants showed the same ability of the wild-type in establishing *in vitro* mycorrhizal symbiosis with the hair roots of *Vaccinium myrtillus* and in influencing plant development. Growth parameters (shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, total leaf area and internodal length) and mycorrhization percentages of *V. myrtillus* seedlings grown with the SOD1-null mutants were recorded after 3 months of co-cultivation and compared to the seedlings inoculated with the wild-type (Table 2). Mycorrhization percentages were also recorded after 1.5 months of co-cultivation (Table 2).

The only statistically significant difference ($P<0.05$) in the plant growth parameters was a slight reduction of the internodal length in the seedlings inoculated with the SOD1-null mutants in respect to those inoculated with the wild-type, even if a high level of variation of this parameter (generally observed on co-cultivation medium) was taken into account. On the other hand, the reduction in the degree of mycorrhization of SOD1-null mutants was always statistically significant ($P<0.05$), both after 1.5 and 3 months.

Table 2. Plant growth parameters and percentages of root colonization of *Vaccinium myrtillus* seedlings mycorrhized with the wild-type *Oidiodendron maius* Zn and the three selected SOD1-null mutants.

	Wild-type	**A4_57**	**D5_125**	**D5_83**
Shoot length (cm)	8.54 ± 1.44	6.34 ± 2.30	5.94 ± 1.61	6.44 ± 2.68
Shoot fresh weight (g)	0.058 ± 0.014	0.051 ± 0.021	0.043 ±0.011	0.047 ± 0.032
Shoot dry weight (g)	0.016 ± 0.004	0.014 ± 0.007	0.012 ± 0.003	0.014 ± 0.009
Root length (cm)	8.64 ± 1.99	7.30 ± 1.96	6.76 ± 1.51	6.20 ± 1.66
Root fresh weight (g)	0.012 ± 0.004	0.011 ± 0.006	0.012 ± 0.003	0.012 ± 0.008
Total leaf area (cm^2)	4.99 ±1.17	4.20 ± 1.66	3.33 ± 0.66	3.71 ± 2.29
Internodal length (cm)	0.34 ± 0.05	0.26 ± 0.04*	0.23 ± 0.04*	0.27 ± 0.06*
% mycorrhization after 1.5 months	4.67 ± 0.32	1.45 ± 0.45*	2.93 ± 0.17*	2.87 ± 0.69*
% mycorrhization after 3 months	6.71 ± 0.84	4.59 ± 1.06*	4.34 ± 1.41*	4.32 ± 1.13*

The results are expressed as the mean ± standard deviation.
All the plant growth parameters were recorded after 3 months.
Asterisks indicate statistically significant differences ($P<0.05$) with the wild-type.

4. Discussion

4.1. First targeted gene disruption in a mycorrhizal fungus

Although mycorrhizae are critical elements of terrestrial ecosystems, we have just begun to understand the molecular interactions between mycorrhizal fungi and their host plants. Since the first global gene expression data from ectomycorrhizas (Johansson *et al*., 2004; Voiblet *et al*., 2001), the genome sequencing program of *Laccaria bicolor* (Martin *et al*., 2008) combined with powerful bioinformatics tools has had a major impact on research on mycorrhizas. Comparison of the genomes of various pathogenic and saprobic fungi with the *Laccaria* genome will enable functional genomic studies with a focus on the complexity of plant-fungus associations (Martin *et al.*, 2007).

The complete inactivation of a gene is generally the first and most direct way to explore its function, but gene manipulation in mycorrhizal fungi is limited by the lack of effective methods for targeted gene replacement on a large scale and the high frequency of ectopic integrations of the transforming DNA molecule.

In this paper we have described the first targeted gene replacement by *Agrobacterium*-mediated transformation in a mycorrhizal fungus. The GR frequency of 1.52% obtained in *O. maius* with both flanking regions of about 800 bp is only slightly lower than the GR frequency observed in the non-mycorrhizal fungus *Aspergillus awamori* (1.75% with flanking regions of 500 bp and 4.04% with flanking regions of 1000 bp) (Michielse *et al*., 2005), but it is very much lower than the GR frequencies observed in *A. niger* (7% with 500 bp and 19% with 1000 bp) *(Meyer et al*., 2007) or *Neurospora crassa* (9% with 500 bp and 21% with 1000 bp) (Ninomiya *et al*., 2004) wild-type strains. Unfortunately, the genome of *O. maius* has not been sequenced, so the length of the flanks we used in the disruption cassette was the longest available based on the known sequence of the *OmSOD1* gene. A significant increase in the frequency may be achieved either by increasing the length of flanking regions or with the suppression of the Non-Homologous End-Joining (NHEJ) pathway, as observed in other filamentous fungi (Meyer *et al*., 2007; da Silva Ferreira *et al*., 2006; Poggeler and Kuck, 2006; Ninomiya *et al*., 2004).

RNA silencing could be a reliable alternative to disruption experiments for the functional analysis of genes, especially in fungi having multinuclear hyphae or a low frequency of homologous recombination (Janus *et al*., 2007). This method has been recently used in the dikaryotic ectomycorrhizal fungus *L. bicolor* to suppress the expression of the nitrate reductase (Kemppainen *et al*., 2009). The RNA silencing of this gene resulted in fungal strains severely affected in their capacity to establish a symbiotic interaction with *Populus*.

Investigations on specific genes mediating symbiotic events in mycorrhiza formation have hitherto been based exclusively on the study of non-mycorrhizal (Myc-) plant mutants interacting with AM fungi (Parniske, 2004). On the fungal side, a collection of ten *Hebeloma cylindrosporum* Myc- mutants blocked at the early stages of ectomycorrhiza formation was obtained by Combier and colleagues (2004). Although the identification of which fungal genes were mutated to give this kind of phenotype was not undertaken, this result, along those reported by Keimpannen *et al*. (2009), confirmed the idea that genes essential for symbiosis establishment do exist in mycorrhizal fungi.

Martin and colleagues have recently used *Laccaria bicolor* whole-genome expression oligoarrays to demonstrate that numerous genes are differentially expressed during the symbiotic stage (Martin *et al.*, 2008). The feasibility of targeted gene disruptions in a mycorrhizal fungus opens new possibilities to study the biological role of these symbiosis-regulated genes. These genes are in fact good candidates for being the primary targets for gene inactivation.

4.2. SOD1 and oxidative stress

Superoxide dismutase enzymes scavenge radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen, thus being involved in the cellular defence strategies against oxidative damage by reactive oxygen species (ROS). Their induction by zinc, cadmium and menadione has been described in other fungi, such as *A. niger* (Todorova *et al*., 2007), *Candida intermedia (Fujs et al*., 2005), *A. nidulans* (Guelfi *et al*., 2003), *C. albicans* (Hwang *et al*., 2002), *Cryptococcus neoformans* (Narasipura *et al*., 2002). The loss of SOD1 activity has various pleiotropic consequences on organisms, which include slow growth, conditional auxotrophies and DNA damage (Fridovich, 1995; Fridovich, 1989). For example, a SOD1-null yeast strain was shown to be oxygen-sensitive, hypermutable, auxotrophic for lysine and methionine and defective in sporulation (Liu *et al*., 1992). It is thus possible that each superoxide dismutase increases fitness, while not being essential for viability (Chary *et al*., 1994).

As expected, *O. maius* Zn SOD1-null mutants showed an increased sensitivity to all the tested substances in respect to the wild-type. Measurement of mycelium radial growth demonstrated that the disruption of *SOD1* caused the mutants to be more sensitive especially to menadione. This quinone, which undergoes intracellular redox cycling, generates a direct oxidative stress and, thus, it is thought to exert its toxicity in a different way from non-redox active substances, such as Zn and Cd, as demonstrated by a large-scale expression experiment on *S. cerevisiae* (Thorsen *et al*., 2009).

Moreover the mutants showed a significant reduction in the number of conidia per surface unit on control medium. A similar result was obtained in a study on the capacity for conidiation of *N. crassa* SOD1-null mutants (Belozerskaya and Gessler, 2006). In the same fungus, SODs were shown to be required for the germination of conidia and for a long lifespan (Munkres, 1992).

Martino and colleagues (2002) have previously demonstrated that *O. maius,* as other eukaryotes, also features a Mn-superoxide dismutase (OmSOD2). Mn-SODs characterized in other fungi are generally located in mitochondria, while the Cu, Zn-SODs usually occur in the cytosol. Although both enzymes catalyse the disproportionation of superoxide to oxygen and hydrogen peroxide, their location in different cellular compartments has been shown to result in independent and unique roles in *Cryptococcus neoformans* variety *gattii* (Narasipura *et al*., 2005) and in *S. cerevisiae* (Dziadkowiec *et al*., 2007). The increased sensitivity of the SOD1-null mutants to all the tested substances in respect to the wild-type supports the hypothesis that Mn-SODs and Cu,Zn SODs have distinct roles in protecting cells from the damaging reactions caused by ROS, that are presumably dismutated in the compartment in which they are generated. Therefore, although OmSOD2 is active in the mutants (data not shown), it cannot compensate for the lack of the OmSOD1.

It is noticeable that strong staining by NBT was observed in the mutants with no appreciable differences between control and stressed hyphae. This result supports the idea that, although viable in atmospheric oxygen, SOD1-null mutants exhibit markers of oxidative stress even during normal cellular growth (Reddi *et al*., 2009; Pereira *et al.,* 2003) and the NBT staining cannot distinguish from those caused by external superimposed stress.

4.3. SOD1 and mycorrhization

SODs are shown to be important not only as anti-oxidant enzymes, but they also play an additional role in fungal morphogenesis and in both the pathogenic and symbiotic interactions between fungi and other organisms (Scott and Eaton, 2008). ROS are strongly implicated in oxidative damage but, paradoxically, small amounts of these reactive species have proven to be involved in important physiological functions, such as signal transduction associated with the control of gene expression and cell proliferation (Brar *et al*., 1999).

Once fungal pathogens have infected a host, they inevitably encounter ROS produced by host cells as well as ROS produced as a consequence of their own oxygen metabolism. During the oxidative burst, SODs play a protective role by suppressing oxidative killing by the infected host. This up-regulation of fungal ROS-scavenger machinery during the early stages of the pathogenic interaction has been demonstrated in *C. albicans* (Hwang *et al*., 2002),

Cryptococcus neoformans (Cox et al., 2003) and *Colletotrichum acutatum* (Brown *et al*., 2008).

A host oxidative burst-like defence response, commonly observed when the plant is confronted with a pathogen, was also detected in compatible mycorrhizal associations (Garcia-Garrido and Ocampo, 2002). Thus, even during symbiosis, a dialogue based on ROS modulation occurs between the fungus and its host plant. Tanaka and colleagues described a role for NADPH oxidases (Nox)-generated ROS in regulating and maintaining the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloe festucae*, and its grass host, *Lolium perenne* (Tanaka *et al*., 2006). This dialogue is essentially based on the superoxide anion which is rapidly converted to hydrogen peroxide either by spontaneous dismutation and or by the catalytic activity of SOD.

Treatment of suspended cells of *Picea abies* (Norway Spruce) with elicitors released from the ECM fungi *Amanita muscaria*, and *Hebeloma crustuliniforme* suggested a rapid but transient production of ROS, mainly H_2O_2 (Salzer *et al*., 1996). Baptista and colleagues evaluated ROS production and the activities of plant superoxide dismutase and catalase during the early contact of the ectomycorrhizal fungus *Pisolithus tinctorius* with the roots of *Castanea sativa* (Baptista *et al.*, 2007). In arbuscular mycorrhizal (AM) associations, a stimulation of fungal respiratory activity and the concomitant induction of ROS-scavenging enzymes have also been reported (Lanfranco *et al*., 2005; Fester and Hause, 2005; Tamasloukht *et al*., 2003; Salzer *et al*., 1999). In particular, an increased expression of *SOD1* was registered in *Glomus intraradices* during the root exudates perception by the fungus (Seddas *et al.*, 2009).

The ability of both pathogenic and symbiotic fungi to maintain this redox dialogue with host plants is based on various ROS-scavenging systems, including superoxide dismutases, catalases, peroxidases, glutathione peroxidases and peroxiredoxins, that, collectively, have an important role in ensuring a rapid turnover of the ROS homeostasis and fine-tuning the ROS-dependent communication between fungi and plants (Scott and Eaton, 2008). How ROS signal within the cell and trigger differentiation processes still remains a key question.

The significant decrease in the percentage of mycorrhization in SOD1-null mutants observed in this work provides further evidence that SOD1 is involved in the fungal morphogenetic responses to the symbiotic partner. In our case, the lack of the SOD1 protein is proposed to cause an imbalance in the redox homeostasis during host colonization and an alteration in the delicate dialogue between *O. maius* and its host plant.

Summarizing, the most important technical advance in our study was the first successful disruption of a gene by homologous recombination in a mycorrhizal fungus. Besides assessing the percentage of homologous recombination in *O. maius* and characterizing the phenotype of

SOD1-null mutants, we have also provided further insights into the role of SOD1 as an important enzyme in the relationship between *O. maius* and its symbiotic partner**.**

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Internet resources

The Tuber melanosporum database
http://mycor.nancy.inra.fr/IMGC/TuberGenome/

Department of Energy Joint Genome Initiative Glomus intraradices page
http://www.jgi.doe.gov/sequencing/statusreporter/psr.php?projectid=16063

Chapter 3

Identification and characterization of genes encoding metal transporters of the CDF and iron permease families conferring zinc tolerance in the metal tolerant ericoid mycorrhizal fungus *Oidiodendron maius*

Abstract

Full-length cDNAs encoding two transporters (*OmCDF* and *OmFET*) were isolated by a yeast functional screening from the ericoid mycorrhizal heavy metal tolerant strain *Oidiodendron maius* Zn. OmCDF belongs to the Cation Diffusion Facilitator (CDF) family whereas OmFET belongs to the iron permease family. Yeast complementation experiments were performed to analyze their functional properties. The heterologous expression of *OmCDF* and *OmFET* in various yeast mutants conferred resistance to zinc. Additionally, *OmCDF* expression also conferred Co tolerance. The GFP fusion proteins revealed that OmCDF were targeted to the endoplasmic reticulum membrane, a result consistent with a function for OmCDF in zinc sequestration. The GFP fusion proteins with OmFET were localized on the plasma membrane. *OmFET* restored the growth of uptake-defective strains for Zn, Fe and Mn. OmFET may acts as an important pathway for Fe uptake in cells exposed to Zn stress.

1. Introduction

Transition metal ions such as iron, copper, manganese and zinc are essential micronutrients that play pivotal roles in several biochemical processes (Waldron *et al.*, 2009). Zinc, in particular, has both a catalytic and a structural function in more than 300 enzymes, belonging to the six major functional enzyme classes (Vallee and Auld, 1990). Although being an essential nutrient, zinc can become toxic if accumulated in excess. Zinc toxicity mechanisms are still poorly unknown. Competitions for catalytic sites and for transporter proteins have been reported as being potentially responsible for the toxicity caused by zinc excess (Eide, 2003). To maintain concentrations of this metal ion within physiological thresholds, organisms have evolved metal homeostasis mechanisms to regulate zinc uptake, distribution, and storage (Eide, 2006). In eukaryotes, zinc homeostasis is largely attributed to the coordinated action of two transporter families: the ZIP (ZRT- IRT-like Protein) and the CDF (Cation Diffusion Facilitator) families (Eide, 2006). A number of ZIP and CDF transporters have been characterized in various organisms (mammals, yeast, nematode, plants) and with the increasing number of genome sequencing projects many candidate ZIP and CDF genes have been identified (Diffels *et al*., 2006; Bolchi *et al*., 2011; Eide, 2006; Kambe *et al*. 2006; Kiranmayi and Mohan 2006; Migeon *et al*., 2010; Montanini *et al*., 2007).

The name of the ZIP transporters family derive from the first two members that were described: the Zrt1 zinc transporter from *Saccharomyces cerevisiae* (Zhao and Eide, 1996a; b) and the Irt1 iron transporter from *Arabidopsis thaliana* (Eide *et al*., 1996). A key feature of the ZIP family is that these proteins transport zinc and/or other metal ions from the extracellular space or organellar lumen into the cytosol (Eide, 2006). The CDF transporter family was first described in *S. cerevisiae* (Zrc1) (Kamizono *et al*., 1989) and in the Gram-negative bacteria *Ralstonia metallidurans* (CzcD) (Nies, 1992). They have also been found in plants and animals (see in Montanini *et al*., 2007). The key feature of the CDF family is that they export zinc and/or other metal ions out of the cells or into intracellular organelles (Eide, 2006; Montanini *et al*., 2007), thus reducing cytosolic metal concentration for detoxification or storage purposes (Anton *et al*., 1999; MacDiarmid *et al.*, 2002). The yeast transcription factor Zap1 is responsible for the regulation of zinc homeostasis at the transcriptional level, controlling the expression of both ZIP transporters, such as ZRT1, ZRT2, ZRT3, and CDF transporters, e.g. ZRC1 under low zinc concentration (MacDiarmid *et al*., 2003).

Much of the current knowledge on zinc homeostasis in fungi comes from the study of the yeast *S. cerevisiae*, used also as a model organism for the identification of several zinc transporters from plants and animal systems. Four transporters involved in zinc uptake have

been reported for *S. cerevisiae*: (a) ZRT1, a high affinity transporter, highly expressed at low external zinc concentrations (Zhao and Eide, 1996a); (b) ZRT2, a low affinity transporter, involved in zinc acquisition under much higher zinc concentrations (Zhao and Eide, 1996b); (c) FET4, a transporter involved in the low affinity uptake of iron, copper and zinc (Waters and Eide, 2002); (d) PHO84, a high affinity phosphate transporter likely also involved in zinc and manganese uptake (Bun-Ya *et al.*, 1991; Jensen *et al*., 2003). Concerning Zn efflux out of the cytosol in *S. cerevisiae*, several CDF transporters play essential roles. ZRC1 and COT1 are responsible for the vacuolar delivery of Zn, and Co and Zn, respectively (MacDiarmid *et al*., 2000; 2002). ZRC1 is involved in Zn homeostasis by providing Zn to the vacuolar alkaline phosphatise under steady-state conditions (Qiao *et al*., 2009). However, ZRC1 and COT1 are the major determinants of Zn tolerance in yeast (Gaither and Eide, 2001). Two other CDF members, MSC2 and ZRG17, form a heteromeric zinc transport complex in ER membranes, and have been implicated in the homeostatic maintenance of ER function (Ellis *et al.*, 2004; 2005).

Despite the importance of zinc as an essential micronutrient, information about the mechanisms of zinc homeostasis and tolerance in fungi other than yeast is still very scarce. However three CDF proteins from other fungal species were characterized. The Zhf1 protein from *Schizosaccharomyces pombe* is involved in the transport of zinc across the membrane of the ER for subsequent zinc storage in this compartment and detoxification of the cells (Clemens *et al.*, 2002). Similarly HcZnT1, from the ectomycorrhizal fungus *Hebeloma cylindrosporum*, is localized on the ER membrane and conferred tolerance when expressed in yeast (Blaudez and Chalot, 2011). Although its role in zinc tolerance could not have been demonstrated, GiZnT1 could be involved in zinc homeostasis in *Glomus intraradices* (González-Guerrero *et al*., 2005) and represents up to now the only transporter gene involved in zinc homeostasis in an endomycorrhizal fungus.

The aim of the present work was to identify and to characterize genes involved in zinc tolerance in the heavy metal tolerant ericoid mycorrhizal fungus *Oidiodendron maius* strain Zn (Martino *et al.*, 2000; 2003). *O. maius* (Barron, 1962) is an ascomycete (class Leotiomycetes) that can establish endomycorrhizal symbioses with the roots of ericaceous plants (Perotto *et al*., 1996; Hambleton *et al.*, 1998; Chambers *et al.*, 2000). Among fungi, mycorrhizal species constitute an important community in metal polluted soils, as they form mutualistic symbioses which can alleviate the effects of heavy metal toxicity to their host plant (Adriaensen *et al.*, 2005; 2006) and provide a more balanced access to mineral elements, either by improving supply of essential elements or by reducing relative uptake of toxic elements (Marschner and Dell, 1994). *O. maius* strain Zn was isolated in the Niepolomice

forest (Poland) from the roots of *Vaccinium myrtillus* plants growing on heavy metals contaminated plots (Turnau, 1988). The colonization of these plots by ericoid mycorrhizal plants suggests that *O. maius* can confer to the host plant the ability to survive in metal polluted environments.

We report here the first transporters involved in Zn tolerance described so far in an ericoid mycorrhizal fungus. More particularly we carried out a functional screening of an *O. maius* cDNA library in the Δ*zrc1* mutant strain of *S. cerevisiae* to search for genes conferring Zn tolerance to yeast cells. Here we describe the identification and characterization of *O. maius* CDF (*OmCDF*) and *O. maius* FET (*OmFET*). We provide evidence that *OmCDF* functions as a zinc transporter responsible for relocating cytoplasmic zinc into the endoplasmic reticulum, whereas expression of *OmFET* could counteract Zn toxicity by increasing iron content of cells.

2. Materials and Methods

2.1. Yeast strains and growth media

Yeast strains used in the present study are listed in Table 1. Growth was either in yeast peptone dextrose (YPD) medium or in synthetic defined medium without uracil (SD-ura) containing 1.7 % (w/v) yeast nitrogen base without amino acids and ammonium, 2% (w/v) glucose or galactose, 5 % (w/v) ammonium sulphate and 1.92 % (w/v) yeast synthetic drop-out medium supplement without uracil (Sigma). pH was adjusted to 5.8.

Table 1. Yeast strains used in this study

Test	Strain	Function of the deleted gene(s)	Genotype
	BY4741	-	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*
	DY1457	-	*MATα; ura3-52; leu2-3,112; trp1-1; his3-11; ade6-1; can1-100(oc)*
Metal tolerance	Δ*ccc1*	Vacuolar Fe and Mn transporter	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; CCC1::kanMX4*
	Δ*cot1*	Vacuolar Zn and Co transporter	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; COT1::kanMX4*
	Δ*pmr1*	Golgi Mn and Ca ATPase	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; PMR1::kanMX4*
	Δ*yap1*	bZIP transcription factor	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YAP1::kanMX4*
	Δ*zrc1*	Vacuolar Zn transporter	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; ZRC1::kanMX4*
Metal uptake	Δ*ctr1*/Δ*ctr3* (MPY17)	Plasmalemma high-affinity Cu transporters	*MATα; ura3-52; his3-Δ200; trp1-901; gal1; CTR1::URA3::Knr CTR3::TRP1*
	Δ*fet3*/Δ*fet4*	Plasmalemma high affinity Fe-uptake system	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR058w::kanMX4; YMR319c::kanMX4*
	Δ*smf1*	Plasmalemma high-affinity Mn transporter	*MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; SMF1::kanMX4*
	Δ*zrt1*/Δ*zrt2* (ZHY3)	Plasmalemma high / low-affinity Zn transporters	*MATα; ade6; can1; his3; leu2; trp1; ura3; ZRT1::LEU2 ZRT2::HIS3*

2.2. Yeast transformation

Yeast transformation was performed using a lithium acetate-based method (Gietz *et al*., 1992). Yeast strains were inoculated into 50 ml YPD medium and incubated overnight at 30°C. Yeast cultures were harvested when an OD_{600nm} of 0.6 was reached. The cultures were centrifuged at 4000 rpm for 5 min. The cells were washed twice with 1 ml sterile water and twice with 1 ml LiAc/TE solution (10 mM Tris-HCl, 1 mM EDTA, 100 mM Lithium Acetate, pH = 7.5). For each transformation experiment, 50 µl of the final yeast cell suspension were mixed with 1 µg plasmidic DNA, 50 µg single strand salmon sperm carrier DNA, and 300 µl sterile LiAc/TE solution containing 50% (w/v) PEG 4000. The mix was incubated first at 30°C for 30 min and finally at 42°C for 15 min. The cells were centrifuged at 4000 rpm for 5 min, washed with 1 ml sterile water and finally plated on SD-ura containing plates.

2.3. Library screening

To identify cDNAs involved in Zn tolerance, an *O. maius* cDNA library (Vallino, unpublished data) was used to transform the Zn-sensitive Δ*zrc1* yeast strain. The library was prepared by pooling the RNA extracted from fungal mycelia exposed to a final concentration of 15 µM $CdSO_4$ for 24 h, 4 days and 18 days. Messenger RNAs were reverse transcribed into cDNAs following the manufacturer's recommendations. cDNAs were cloned into the yeast expression plasmid pFL61. The transformation procedure of Δ*zrc1* yeast cells with the cDNA library was the same as that described above, except that after the 42°C shock, the cells were washed with 1 ml of sterile water and incubated in liquid YPD medium for 4 h. Finally cells were plated on SD-ura medium containing 17.5 mM $ZnCl_2$. Among the resistant clones 200 colonies were picked up and further tested on 12.8 mM 5-fluoroacetic acid (FOA) to eliminate false positive colonies. Plasmids were extracted from confirmed positive colonies using the Zymoprep yeast plasmid minipreparation kit (Zymo Research) following the manufacturer's recommendations. Plasmids were subsequently amplified in *E. coli*. As a definitive confirmation of the plasmid-associated Zn resistance of cells, plasmid minipreparations (Promega) were realised and used to transform the Δ*zrc1* yeast mutant and further retested for Zn tolerance. The plasmids that conferred Zn tolerance were sequenced.

2.4. Phenotyping tests

To examine the metal tolerance specificity of the identified cDNAs, a set of *S. cerevisiae* strains was used (Table 1). Wild-type (BY4741) and mutant strains, carrying the empty vector (pFL61), pFL61-*OmFET*, or pFL61-*OmCDF*, were grown on control SD-ura medium, and on the same medium supplemented with either 20 mM $ZnCl_2$, 5.25 mM $FeCl_3$, 2 mM $CoCl_2$, 50 µM $CdCl_2$, and 2 mM $MnCl_2$ for Δ*zrc1*, Δ*ccc1*, Δ*cot1*, Δ*yap1* and Δ*pmr1*, respectively.

To test the metal uptake properties of the OmFET, another set of yeast mutants was also used (Table 1). Wild-type (DY1475) and *ZHY3* (Δ*zrt1*/Δ*zrt2*) strains were grown on YPD medium or on SD-ura medium containing either no extra Zn, 0.5, 1 or 1.5 mM $ZnCl_2$, and supplemented with 1 mM EDTA. The double mutant Δ*fet3*/Δ*fet4* was grown on SD-ura medium supplemented with 0, 50, 100, 200 µM $FeCl_3$, or on YPD medium supplemented with 200 µM $FeCl_3$. Wild-type (BY4741) and Δ*smf1* were grown on SD-ura medium in the presence of 0 or 10 mM ethylene glycol-*bis* (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), or on YPD medium. *MPY17* was grown on the SD-ura medium with the addition of different concentrations (0, 50, 100, 200 µM) of the copper chelator bathophenanthrolinedisulfonic acid (BPS), or on YPD medium. *AtIRT1*, an *Arabidopsis thaliana*-metal ion transporter, was used as a positive control. The *AtIRT1* is considered to be

the main transporter for high-affinity iron uptake in roots (Vert *et al*., 2002), and is reported to be able to also transport zinc, cobalt, manganese and cadmium (Rogers *et al*., 2000; Connolly *et al*., 2002).

2.5. Sequence analyses

Analyses of similarity were performed using the program BLASTX at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence translations were performed using Expasy (http://www.expasy.ch/). Full-length amino acid sequences of orthologs from selected sequenced ascomycetous genomes were obtained from the Broad Institute website (http://www.broadinstitute.org/). For phylogenetic tree construction, sequences were aligned by CLUSTALW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 4 (Tamura *et al*., 2007). Phylogenetic analyses were conducted using the neighbor-joining (NJ) method implemented in (MEGA), with the complete deletion option, and a Poisson correction model for distance computation was used. Bootstrap tests were conducted using 1000 replicates. Branch lengths are proportional to phylogenetic distances. The sequences were also aligned and edited by the multiple sequence alignment editor and shading utility GeneDoc software (version 2.6.003) (http://www.psc.edu/biomed/genedoc). The prediction of the hydropathy profiles was generated with the topology prediction programme TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.6. Construction of GFP fusion proteins for expression in yeast and confocal observations

To construct *OmCDF::GFP* and *OmFET::GFP*, the open reading frame (ORF) of *OmCDF* and *OmFET* were amplified by PCR using the pFL61-*OmCDF* and pFL61-*OmFET* plasmids previously isolated as template. Both forward primers contained *KpnI* sites (KpnOmCDFf 5'-ATCAGGTACCATGGCGTGGTCAAAATCGAC-3' and KpnOmfet4f 5'-ATCAGGTACCATGCAGCGAATAATCAGAGC-3'), while reverse primers contained *NotI* sites (NotOmCDFr (5'-ATATGCGGCCGCTGAACGTGGTCGTTGTCATC-3' and NotOmfet4r (5'-ATATGCGGCCGCGCCACGGGTAGCAATTGACG-3'). The PCR products were *NotI*- and *KpnI*-digested and inserted into the KpnI-NotI-digested pYES2-GFP plasmid (Blaudez *et al*., 2003). Fluorescence emission from cells expressing *OmCDF::GFP* or *OmFET::GFP* was examined with the use of a confocal laser-scanning microscope (Leica) equipped with a TCS SP2 Laser. GFP was excited with a 488 nm laser line and emission fluorescence was detected at 515–530 nm. Images were recorded and processed using Adobe Photoshop 7.0 image software (Sunny Valley, CA, USA). To reveal the nuclear position

within cells, DAPI (4',6-diamidino-2-phenylindole) staining of the cells was realised. A 10 µg/ml DAPI solution was added to yeast cells just before microscopic observation.

3. Results

3.1. Two metal transporters conferring Zn tolerance and belonging to the CDF and to the iron permease family were identified

Approximately 12.5% of the Δ*zrc1* transformants were able to grow on high Zn. Ten clones were finally isolated by this screening. Among them, five clones were highly tolerant and contained a sequence coding for metal transporters. One of them (OmCDF) was found as a single copy from the library screening, whereas the other four clones were identical and encoded the same protein (OmFET). We therefore focused on the functional characterization of these two genes.

Concerning the first clone that was found as a single copy, the *in silico* translation of the full-length cDNA (1686 bp) revealed an open reading frame of 1629 bp encoding a 542 amino acid polypeptide with a predicted molecular weight of 58.7 kDa. Comparisons with protein sequence databases from NCBI showed that it was related to members of the CDF family. More particularly, the analyses revealed that the encoded protein is a close relative to putative CDF proteins of *Sclerotinia sclerotiorum* (XP_001593376) and *Botryotinia fuckeliana* (XP_001545800) (61% and 60% of similarity, respectively). It also showed 55% and 50% of similarity with the previously characterized ZRC1 and COT1 proteins from *S. cerevisiae*, respectively (Li and Kaplan, 1998; Lyons *et al*., 2000), and 55% of similarity with ZHF1 from the fission yeast *Schizosaccharomyces pombe.* The clone was therefore named OmCDF. The hydropathy profile generated with the topology prediction programme TMHMM version 2.0 predicted six transmembrane domains in the deduced amino acid sequence (Fig. 1A). This feature is typical of the majority of the previously described CDFs (Montanini *et al.*, 2007). A histidine-rich motif $(HX)_8$ was also found in the cytoplasmic loop located between helices IV and V. As indicated in the phylogenetic tree of Figure 2, OmCDF belongs to the CDF sub-family I. This sub-family groups CDF proteins transporting Zn as the major substrate. ZRC1, COT1, and ZHF1 also grouped in the same cluster of this sub-family. These data suggest that OmCDF could have a similar role than those of their hemi-ascomycete orthologs.

The second clone that was found in four copies from the library screening consisted in a 1515 bp cDNA fragment. The translation of this sequence revealed an open reading frame of 1515 bp and a corresponding polypeptide of 504 residues with a predicted molecular weight of 56.3 kDa. BLAST searches against protein databases showed that the deduced protein is related to members of the iron permease family. The best hit corresponded to the low affinity Fe^{2+} transporter FET4 of *Aspergillus* (53% of similarity) and subsequent hits also corresponded to putative or characterized FET4 orthologs from ascomycetes. The clone was therefore named

OmFET. The hydropathy profile generated with TMHMM predicted height transmembrane domains (Fig. 1B).

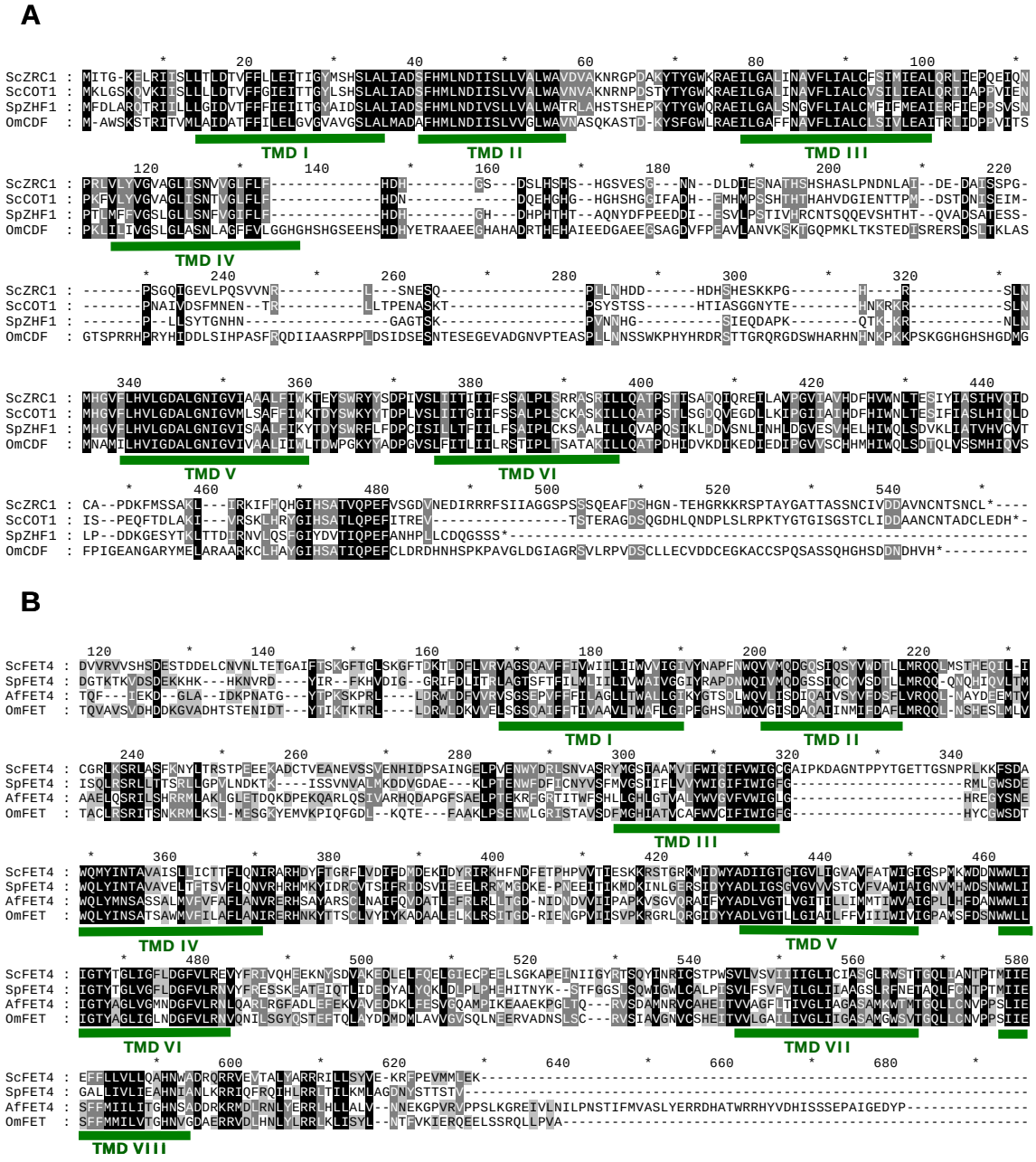

Figure 1. Protein sequence comparison of OmCDF (A) and OmFET (B) with ascomycetous orthologs. Amino acid residues that are conserved in all four sequences, or in at least two of them are shown on a black or grey background, respectively. The positions of predicted transmembrane domains (TMDs) are underlined in green. The reference proteins and their corresponding accession numbers used were: SpZHF1 (NP_593645), ScZRC1 (CAA88653), ScCOT1 (CAA99636), ScFET4 (AAA53129), SpFET4 (NP_595134), and AfFET4 (XP_751369).

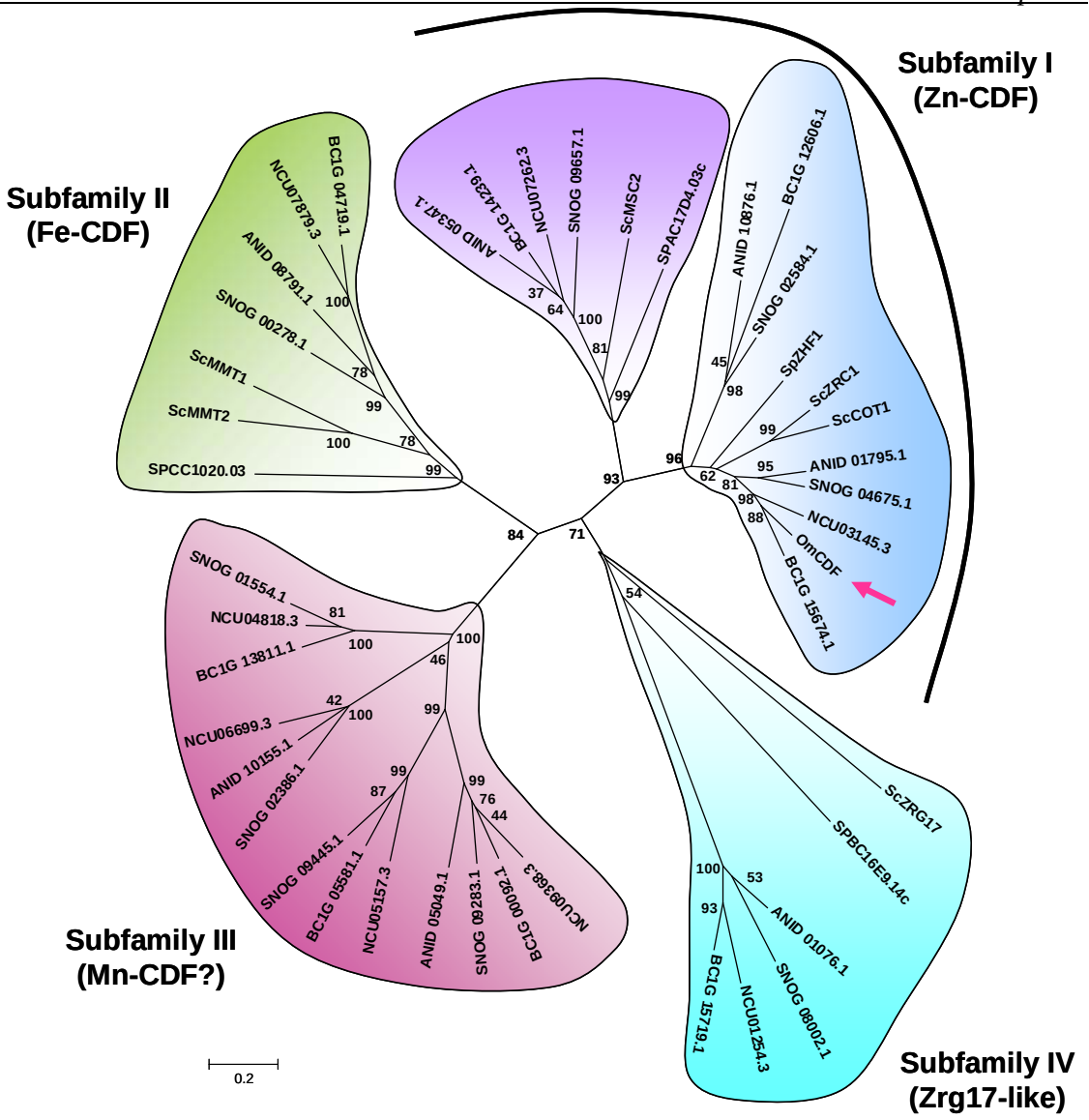

Figure 2. An unrooted, neighbor-joining (NJ)-based tree of the Cation Diffusion Facilitator (CDF) family in selected ascomycetous fungi. The analysis was performed as described in the "Materials and Methods" section and the tree was generated using MEGA version 4 (Tamura *et al.*, 2007) after sequence alignment. Bootstrap values are indicated (1000 replicates). Branch lengths are proportional to phylogenetic distances. The red arrow highlights the position of OmCDF in the tree. Names of the fungal species are abbreviated with the following codes: Sc: *Saccharomyces cerevisiae*, Sp or SP: *Schizosaccharomyces pombe*, ANID: *Aspergillus nidulans*, NCU: *Neurospora crasse*, BC: *Botrytis cinerea*, SNOG: *Stagonospora nodorum*.

3.2. Expression of *OmCDF* and *OmFET* in yeast confers tolerance to Zn

OmCDF and *OmFET* were isolated by the functional screening of the *O. maius* cDNA library in the Zn hypersensitive Δ*zrc1* mutant suggesting that they are both involved in Zn tolerance. To further characterize *OmCDF* and *OmFET*, cDNAs were expressed in *S. cerevisiae* mutant strains that are unable to grow at high concentrations of various metals, and growth was monitored on either control or metal-supplemented media. The chosen mutant strains Δ*zrc1*,

Δ*ccc1*, Δ*cot1*, Δ*yap1* and Δ*pmr1* are unable to grow on Zn, Fe, Co, Cd, and Mn, respectively. ZRC1 encodes a transporter that sequesters Zn into the vacuole: its deletion rendered the mutant highly sensitive to Zn (Li and Kaplan, 1998). *CCC1* encodes a transporter that mediates Fe and Mn accumulation into the vacuole, *ccc1* mutants showed increased sensitivity to external iron (Li *et al*., 2001). *COT1* mediates the efflux of Zn and Co into the vacuole (Lyons *et al*., 2000) and *cot1* mutants are hypersensitive to both Zn and Co. *YAP1* is a transcription factor belonging to the Yap basic-leucine zipper (bZip) family of stress response regulators, it regulates a yeast response to oxidative stress and to metals such as Cd (Azevedo *et al*., 2007). *PMR1* is the yeast secretory pathway pump responsible for Mn and Ca transport into the Golgi and confers Mn tolerance by effectively removing Mn from the cytosol (Ton *et al*., 2002).

As described above, the Zn-sensitive phenotype of the *zrc1* mutant was totally abolished when overexpressing OmCDF or OmFET. Both OmCDF and OmFET-expressing Δ*zrc1* strains were indeed as tolerant as the wild-type strain at 20 mM Zn (Fig. 3a) and grow even better at higher concentrations (data not shown). Conversely, growth of the *ccc1* mutant was not restored at 5.25 mM Fe by OmCDF or OmFET (Fig. 3b). Interestingly, the Co sensitive phenotype of the *cot1* mutant was partially abolished by OmCDF, but not by OmFET (Fig. 3c). Expression of *OmCDF* and *OmFET* did not restore cell growth of the *yap1* mutant on 50 µM Cd (Fig. 3d), neither that of the *pmr1* mutant on 2 mM Mn (Fig. 3e). The data obtained with the five mutant strains suggest that OmCDF and OmFET conferred tolerance to Zn, and additionally, that OmCDF also conferred tolerance to Co. No significant improved tolerance to Fe, Cd, or Mn could be found.

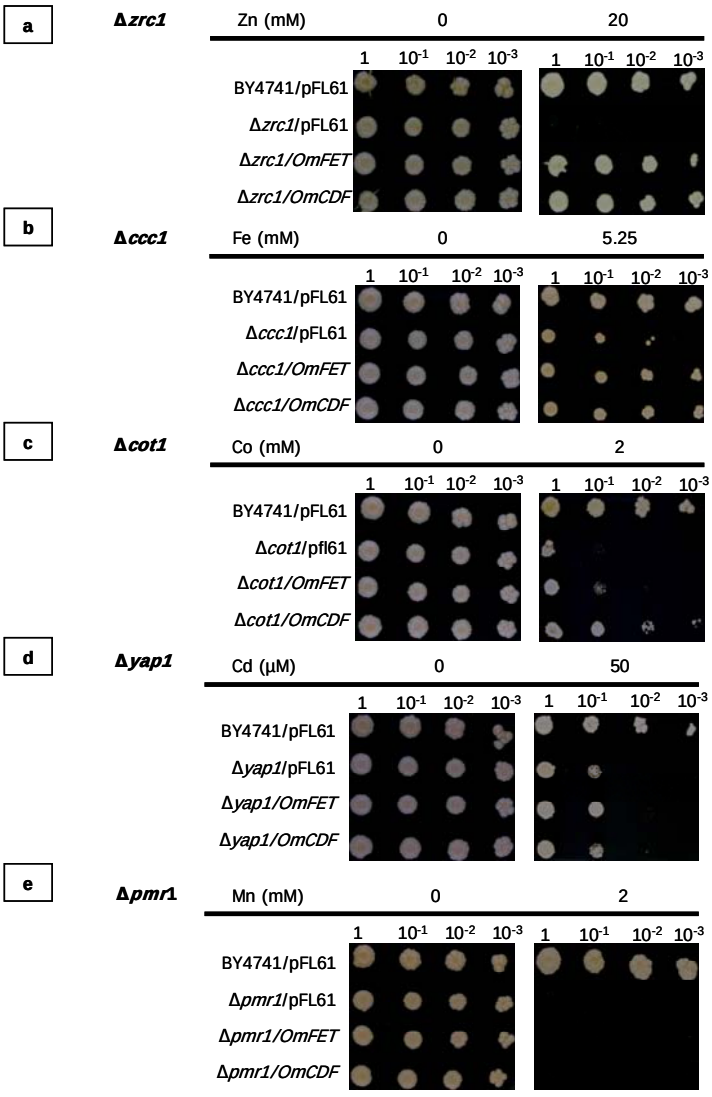

Figure 3. Functional complementation of metal-sensitive yeast strains by *OmFET* and *OmCDF*. Cultures of WT (*BY4741*) and mutant strains were tenfold serial diluted and spotted on either control or metal-supplemented SD-ura medium. The WT strain (BY4741) was transformed with the empty vector pFL61. The mutant strains were transformed either with the empty vector pFL61, pFL61-*OmFET*, or pFL61-*OmCDF*. Pictures were taken after 3 days of growth and are representative of two separate experiments.

3.3. OmFET is able to rescue the growth of different metal uptake-deficient yeast strains

Homology searches revealed that OmFET was a close relative to FET4 of *S. cerevisiae* (see above). ScFET4, a plasmalemma resident transporter, is primarily involved in the uptake of iron in yeast and could also transport a series of metals including Zn, Mn, and Cd. In order to determine the ability of OmFET to take up metals into the cytosol, we expressed *OmFET* in the *Zhy3* (*zrt1*Δ*/zrt2*Δ), *fet3*Δ*/fet4*Δ and *Smf1*Δ yeast strains, which are unable to take up Zn, Fe and Mn respectively, and growth was monitored on either control or metal-limiting medium (Fig. 4). ZRT1 acts as a high-affinity zinc transporter (Zhao and Eide, 1996a) whereas ZRT2 is a low affinity zinc transporter (Zhao and Eide, 1996b), both belonging to the ZIP transporter family. Therefore the double mutant *zrt1*Δ*/zrt2*Δ is not able to grow on Zn-deficient conditions. The *fet3*Δ*/fet4*Δ strain is an Fe uptake-deficient double mutant, FET3 being a multicopper oxidase belonging to a high-affinity iron transport system (DeSilva *et al*.,

1995) and FET4 being a low affinity iron transporter system, as previously reported. SMF1 functions in the cellular accumulation of Mn, and the *smf1*Δ mutant can not grow on Mn-limiting medium (Supek *et al*., 1996).

Overexpression of *OmFET* restored the ability of the Zhy3 strain to grow on a Zn-limited medium (controlled by the addition of EDTA) at the same level than that of *AtIRT1* transformed cells (Fig.4a). *AtIRT1* was used as a positive control. AtIRT1 is the main transporter for high-affinity iron uptake in Arabidopsis roots, and is also involved in Mn, Zn, and Cd transport when expressed in yeast (Rogers *et al*., 2000; Connolly *et al*., 2002). *OmFET* could also complement the Fe-uptake defective *fet3*Δ/*fet4*Δ strain at the same level than that found with *AtIRT1* (Fig. 4b). Moreover the Mn-sensitive phenotype of the *smf1*Δ mutant was restored by *AtIRT1* and even better by *OmFET* (Fig. 4c). Finally, *OmFET* did not restore cell growth of MPY17, a Cu-uptake deficient strain (Peña *et al*., 1998) under Cu limiting conditions (data not shown). Altogether these data suggest that OmFET could transport Fe, Zn, and Mn, but not Cu.

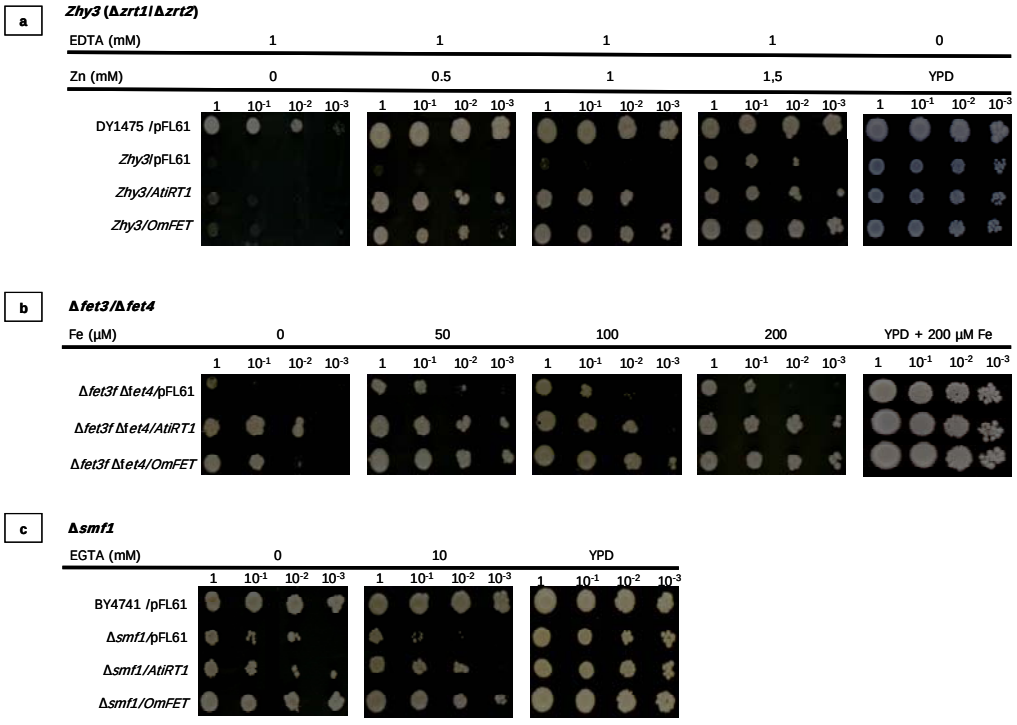

Figure 4. Functional complementation of metal uptake-deficient yeast strains by *OmFET*. Cultures of WT (BY4741 or DY1457) and mutant strains were tenfold serial diluted and spotted on YPD medium, or on either control or metal-limiting SD-ura medium. Low Zn and Mn media were obtained by the addition of 1 mM EDTA and 10 mM EGTA, respectively. The SD-medium is naturally a Fe-limiting medium since the Fe uptake-deficient Δ*fet3*/Δ*fet4* strain could not grow. The WT strains were transformed with the empty vector pFL61. The mutant strains were transformed either with the empty vector pFL61, pFL61-*AtIRT1*, or pFL61-*OmFET*. *AtIRT1* was used as a positive control. Pictures were taken after 3 days of growth and are representative of two separate experiments.

3.4. *OmFET* is targeted to the plasmalemma while *OmCDF* is localized at the endoplasmic reticulum membrane

The *OmFET::GFP* and *OmCDF::GFP* fusion proteins were constructed to determine the sub-cellular localization of *OmFET* and *OmCDF*. The bright-field images in Figure 5A show the clear presence of vacuoles in yeast cells. At the standard image acquisition settings used for GFP visualization, autofluorescence from cells transformed with the untagged genes was absent (data not shown), so all detectable fluorescence in the transformants was GFP specific. Figure 5 shows that, when exponentially growing cells were analyzed by confocal laser scanning microscopy, green fluorescence resulting from *OmFET::GFP* showed a ring pattern surrounding the cell, reflecting a localization on the plasmalemma (Fig. 5A). The GFP-derived fluorescence pattern of *OmCDF::GFP* showed a pattern that typically reflects a localization at the endoplasmic reticulum membrane (Fig. 5B). The fluorescence was noticeably detected around the nuclei, as evidenced by DAPI counterstaining.

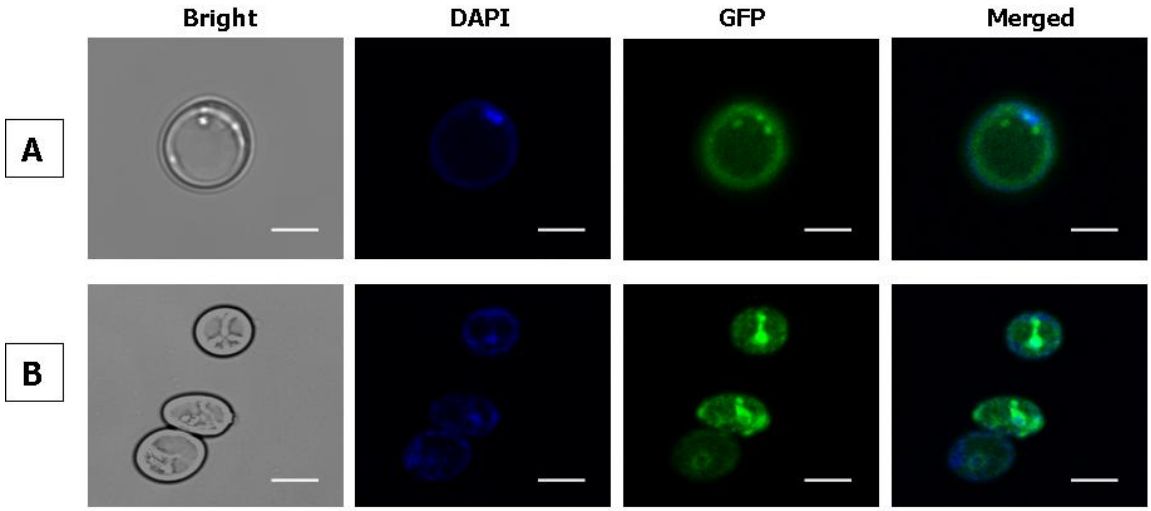

Figure 5. Sub-cellular localization of OmFET::GFP (A) and OmCDF::GFP (B) fusion proteins in yeast by confocal laser microscopy. Cells were grown in SD-ura medium and were stained with DAPI. Different images from the same cells are shown: bright field (first panels), DAPI-derived fluorescence (second panels), GFP fluorescence (third panels). The fourth panels represent the merged pictures of DAPI + GFP images. Bars = 10 µm.

4. Discussion

Metals can be introduced in terrestrial ecosystems through human activities and they may also occur in naturally metalliferrous soils. In both cases these elements can be present at potentially toxic concentrations. Eukaryotic microorganisms have adopted two main strategies for decreasing the toxicity of metals: chelation and efflux from the cytosol, either out of the cell or into cellular compartments (Bellion *et al*., 2006). A number of metal transporters have been characterized in yeast (Kamizono *et al*., 1989; Clemens *et al*., 2002; Fang *et al*., 2008) and filamentous fungi (Kiranmayi *et al*., 2009; González-Guerrero *et al*., 2005; Kosman 2003). In particular Kiranmayi and Mohan (2006) identified 15 different transporter families in the *Neurospora crassa* 'Metal Transportome'.

Zinc is an essential metal fundamental for the structure and/or function of many prokaryotic and eukaryotic proteins, and although oxidatively non-reactive, its excess is deleterious to cells. All organisms have developed homeostatic mechanisms aimed to ensure the minimal zinc levels required for normal metabolism but also to avoid zinc intoxication **(**Pagani *et al.*, 2007). *O. maius* Zn is an ericoid mycorrhizal strain isolated in 1995 from roots of *Vaccinium myrtillus* growing in the Niepolomice Forest (Krakow, Poland), on a plot where Zn was the most abundant heavy metal in the contaminating dust (22% ZnO, 0.63% CdO, 8.13% Al_2O_3) (Martino *et al.*, 2000). An elevated zinc tolerance was previously demonstrated for this strain (Martino *et al.*, 2000; 2002; 2003), but the associated zinc tolerance mechanisms have not been evidenced yet. In the present study we identified *OmCDF* and *OmFET*, two genes encoding transporters involved in Zn tolerance, by screening a cDNA library from *O. maius* in a Zn-sensitive yeast mutant under high Zn concentrations. OmFET belongs to the iron permease family whereas OmCDF belongs to the CDF family. The putative roles of both proteins in Zn tolerance are discussed below.

Proteins belonging to the CDF (Cation Diffusion Facilitator) family have been implicated in the metal tolerance mechanisms of a range of organisms. Indirect evidence suggests that these proteins are transporters that either sequester metal ions within cells or export metal ions out of cells (Paulsen and Saier, 1997). The CDF proteins described to date confer Zn, Cd, Co, Mn or Ni tolerance to a range of organisms, and many are located on internal membranes. In *S. cerevisiae*, the CDF transporters ZRC1 and COT1 are responsible for the vacuolar delivery of Zn, and Co and Zn, respectively (MacDiarmid *et al*., 2000; 2002; 2003). These two transporters have roles in both Zn homeostasis and Zn tolerance. Under sufficient conditions, ZRC1-mediated delivery of Zn in the vacuole is essential for the activity of the vacuolar alkaline phosphatase (Qiao *et al.*, 2009). Moreover under Zn shock, *ZRC1* up-regulation

reduced Zn toxicity of cells facing a switch from Zn-replete to Zn-deficiency conditions (MacDiarmid *et al*., 2003). However most importantly, ZRC1- and COT1-mediated vacuolar storage of Zn are the major determinants of Zn tolerance in yeast (Gaither and Eide, 2001). The ER-resident CDF protein ZHF1 from the other yeast model system, *Schizosaccharomyces pombe*, has been demonstrated to be the major determinant of Zn tolerance in this organism (Clemens *et al*., 2002). Contrary to *S. cerevisiae* where vacuoles ensure Zn storage and detoxification, Zn is mainly stored in the ER in *S. pombe*. Together with the fact that ZHF1 is the unique Zn-CDF found in the genome of this fungus, all these data suggest different strategies for Zn homeostasis/tolerance between the two yeast models.

Less information is available concerning filamentous fungi. In *Glomus intraradices*, González-Guerrero and colleagues (2005) were able to isolate a Zn-CDF transporter from the mycorrhizal glomeromycete fungus (*GintZnT1*). They suggested a role for *GintZnT1* in Zn compartmentalization and although a role in Zn tolerance could not be demonstrated, this transporter might contribute to Zn homeostasis in hyphae. More recently, HcZnT1 from the basidiomycete fungus *Hebeloma cylindrosporum* was functionally characterized. A HcZnT1::GFP fusion protein was localized to the ER membrane and HcZnT1 was able to specifically confer Zn tolerance when expressed in various yeast mutants (Blaudez and Chalot, 2011). *OmCDF* encodes a protein which has been assigned to the CDF family of zinc transporters based on examination of its sequence and general structural features. In fact, most CDF family members are predicted to have six transmembrane domains and two cytoplasmic loop regions (Gaither and Eide, 2001). The same characteristics were observed for *OmCDF*, together with the close sequence relationship between *OmCDF* and other characterized CDF members from hemiascomycetes, suggesting a role for this protein in the homeostasis and/or tolerance of Zn in *O. maius*. We assessed *OmCDF* function in *S. cerevisiae*. In the present study expression of the OmCDF protein restored the growth of metal-sensitive phenotypes of several yeast mutants. In particular it was able to restore the growth of Zn- and Co-sensitive mutants, but was unable to complement the Cd- and the Mn-hypersensitive phenotypes. These data suggest that OmCDF is specifically involved in Zn and Co tolerance.

Localization studies suggest that *OmCDF::GFP* is targeted to the ER membrane and could therefore mediate zinc delivery into this compartment. Zn-CDF members have a range of different sub-cellular localizations, including on most intracellular membranes and the plasma membrane. As stated above, some studies reported the same ER localization as that found for *OmCDF*. In *S. cerevisiae*, two other CDF family members, MSC2 and ZRG17, form a heteromeric zinc transport complex in the ER membranes, and they have been implicated in the homeostatic maintenance of ER function (Ellis *et al.*, 2004; 2005). As depicted on Figure

2, MSC2 and ZRG17 are not orthologs of OmCDF. On the contrary the ER-resident SpZHF1 and HcZnT1 (Blaudez and Chalot, 2011) group together with OmCDF within a same cluster of the Zn-CDF subfamily. Taken into consideration both the results from metal tolerance tests and from the subcellular localization of *OmCDF*, we hypothesize a role of OmCDF in the ER transport of Zn and a contribution in Zn detoxification as observed in *S. pombe* (Clemens *et al*., 2002). However, Zn-CDF transporters localized on the vacuolar membrane could also be present in *O. maius*. The coming genome sequencing of *O. maius* should reveal the presence or absence of other putative Zn-CDFs in this organism. As a conclusion, OmCDF is the first transporter described in Zn tolerance in a filamentous ascomycete, as well as in an ericoid mycorrhizal fungus.

The second clone that was isolated from the Zn tolerance screening encodes OmFET. BLAST analyses revealed that OmFET belongs to the iron permease transporter family. Close orthologs of OmFET comprise the proteins FET4 from *S. cerevisiae* and *S. pombe*, and FETD from *A. nidulans*. Yeast can use a high-affinity reductive mechanism for iron uptake. Extracellular Fe(III) is reduced to Fe(II) and subsequently used as substrate by the high affinity transport system composed of the *FET3* multicopper oxidase and the *FTR1* permease (Waters and Eide, 2002). In addition to the high affinity system, low affinity iron uptake systems are also present in yeast. This is evident because mutational inactivation of the high affinity system does result in viable cells. The low affinity iron transporter is encoded by the *FET4* gene (Dix *et al*., 1994; 1997). The FET4 protein has six predicted membrane spanning regions and is localized to the plasma membrane (Waters and Eide, 2002). The *FET4* gene, found in ascomycete fungi such as *S. cerevisiae, S. pombe, Candida albicans* and *Aspergillus fumigatus*, was originally identified only as a low-affinity iron transporter (Dix *et al*., 1994). However it has been demonstrated that *FET4* has a broad specificity and it can transport other metals. The *S. cerevisiae* FET4 protein acts as a zinc (Waters and Eide, 2002) and also as a copper transporter (Hassett *et al*., 2000). Li and Kaplan (1998) and Pagani *et al*. (2007) attributed the increase in intracellular zinc content in iron depleted *S. cerevisiae* cultures to the low-affinity iron transporter FET4, which also transports copper and zinc.

Similarly, Dainty *et al.* (2008) reported that the FET4 ortholog of *S. pombe* can transport zinc. In addition, FETD, the FET4 ortholog of *A. fumigatus*, was involved in the accumulation of zinc during iron starvation (Yasmin *et al*., 2009). *FET4* has also been shown to be a cobalt and manganese transporter (Li and Kaplan, 1998). *FET4* plays therefore a central role in the uptake of a number of metal ions. Coupled with the fact that the OmFET::GFP fusion protein was detected at the plasma membrane, all these data prompted us to investigate the metal transport capability of OmFET. For this purpose, we first expressed *OmFET* in the

Δ*fet3*/Δ*fet4* yeast strain that can not grow under limiting Fe conditions. Overexpression of OmFET restored the mutant growth, strongly suggesting that OmFET is a transporter delivering Fe to the cytoplasm. Then, in order to test the transport specificity of OmFET, other metal uptake-deficient strains were also used. The *zhy3*, Δ*smf1*, and MPY17 strains can not grow under Zn, Mn, and Cu limiting conditions, respectively. Similarly to AtIRT1, used in the present study as a positive control, OmFET restored the growth of the mutant strains under limiting Zn and Mn conditions, but not under low Cu conditions. Therefore the data from the present study indicate that OmFET could transport Fe, Zn, and Mn.

The second series of results with OmFET when expressed in yeast concerns the specificity of metal tolerance. Contrary to OmCDF, OmFET-associated tolerance was Zn specific. Expression of OmFET did not indeed restored the growth of sensitive yeast mutants on high concentrations of Co, Cd, Cu, Fe, or Mn. Cells require finely tuned homeostatic mechanisms to balance uptake and storage of Zn and a highly regulated coordination of zinc and iron metabolisms has been reported in the literature (Yasmin *et al*., 2009). An interconnection between zinc and iron metabolisms has been reported for *S. cerevisiae* by Pagani *et al*. (2007). Interestingly, in the screening of a systematic deletion mutant library for altered growth in the presence of zinc, a mutant lacking the Aft1 transcription factor, required for the transcriptional response to iron starvation, was found to be highly sensitive to zinc. Exposure to zinc resulted in rapid increase in the expression of some components of the iron regulon, as revealed by a genome-wide transcriptional profiling (Pagani *et al*., 2007). Interestingly, zinc excess resulted in decreased intracellular iron content and aconitase and cytochrome c activities. These findings further suggested that high zinc levels may alter the assembly and/or function of iron-sulphur-containing proteins, as well as the biosynthesis of heme groups, thus establishing a link between zinc, iron and sulphur metabolism. Li and Kaplan (1998) also found that the loss of high-affinity iron uptake in yeast resulted in increased sensitivity to zinc. A genomewide, microarray-based transcription profiling in *A. fumigatus* has indicated that expression of various genes encoding zinc homeostasis-maintaining proteins is affected by iron availability (Schrettl *et al*., 2008). Yasmin *et al*. (2009) clearly showed that iron has an effect on the expression of zinc transporters. They observed an increased Zn content during iron depleted compared to iron replete conditions. This observation suggested that iron starvation induced the expression of other transporters, which mediate the uptake of zinc in addition to iron. In particular, Yasmin *et al*. (2009) showed that iron starvation affects zinc metabolism by down-regulating expression of the plasma membrane zinc importer encoding zrfB and up-regulating the putative vacuolar zinc transporter-encoding zrcA in *Aspergillus fumigatus*. The increase of zinc uptake by overexpression of zinc transporters was more toxic

under iron depleted compared to iron replete conditions. Moreover, the zinc content of iron-starved mycelia exceeded that of iron replete mycelia, possibly due to unspecific metal uptake induced by iron starvation. Deficiency in siderophore-mediated high-affinity iron uptake caused hypersensitivity to zinc and this is in agreement with increased zinc excess and zinc toxicity during iron starvation. Taken together, these data demonstrate a fine tuned coordination of zinc and iron metabolisms in *A. fumigatus*. From the above findings we can therefore conclude that zinc sensitivity can be an outcome of disruption of iron homeostasis that creates an imbalance in cellular zinc and iron contents (Yasmin *et al*., 2009). As our experiments showed that *OmFET* confers resistance to zinc we can speculate that this could be due to its ability to mediate iron accumulation under zinc stress, consequently counteracting zinc toxicity. To test this hypothesis cultures with different concentrations of zinc and iron should be prepared and analysed. More generally, this overlap between the metabolism of diverse metals in fungi suggests the existence of metallomic networks underlying many cellular responses, recently confirmed at the genomic scale (Eide *et al*., 2005).

González-Guerrero *et al*. (2005) cloned and analysed the regulation of a *Glomus intraradices* Zn transporter, as a first step to gain insights into zinc homeostasis mechanisms in arbuscular mycorrhizal (AM) fungi, ubiquitous soil-borne microorganisms that establish a mutualistic symbiosis with most plant species. Under conditions of high available Zn, the concentration of this element is lower in mycorrhizal than in non-mycorrhizal plants (Bi *et al*., 2003; Joner and Leyval, 1997). Considering the importance of the dual role of mycorrhizal fungi in plant nutrition and in metal toxicity avoidance, the understanding of metal homeostasis/tolerance mechanisms in symbiotic mutualistic fungi is of fundamental importance. *O. maius* strain Zn was isolated in the Niepolomice forest (Poland) from the roots of *Vaccinium myrtillus* plants growing on heavy metal contaminated plots (Turnau, 1988). The colonization of these plots by ericoid mycorrhizal plants suggests that *O. maius* can confer to the host plant the ability to survive in metal polluted environments. The comprehension of Zn homeostasis mechanisms in *O. maius* Zn could help to explain the role played by this fungus in the tolerance behaviour of the host plant. Here we show that different transporters might participate in *O. maius* Zn tolerance. Zn transporters extruding excess Zn out of the cytosol and improvement of Fe uptake by cells are two important pathways for detoxifying hyphae against Zn stress.

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Internet resources

BLASTX

http://blast.ncbi.nlm.nih.gov/Blast.cgi

Expasy

http://www.expasy.ch/

Broad Institute website

http://www.broadinstitute.org/

GeneDoc software (version 2.6.003)

http://www.psc.edu/biomed/genedoc

Topology prediction programme TMHMM version 2.0

http://www.cbs.dtu.dk/services/TMHMM/

Chapter 4

Screening of a random-mutant collection of *Oidiodendron maius* and identification of genes potentially involved in oxidative stress tolerance

Abstract

Insertional mutagenesis is a powerful tool to screen for a phenotype of interest and to assign a biological function to the mutated gene. A collection of 1087 random mutants of the metal-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn, obtained through random integration of an hygromycin cassette, was used to screen for metal and/or oxidative stress sensitive mutants and to eventually identify genes involved in the stress tolerance mechanisms. Twenty three out of the 309 screened mutants showed an increased sensitivity, with respect to the wild-type, to at least one of the three tested substances: zinc, cadmium and menadione. Using Thermal Asymetric Interlaced (TAIL)-PCR we identified the mutated genes for three of these mutants. The three genes coded for: a member of the major facilatator superfamily (MFS) proteins, a bZIP transcription factor, and a nitrogen metabolite repression (*nmr*) gene.

1. Introduction

Oxidative stress is a common phenomenon triggered by several environmental stresses. Recently, Bressano *et al*. (2010) highlighted the importance of mycorrhizal fungi in oxidative stress regulation as a general strategy to protect plants from abiotic and biotic stress. Among oxidative stress causing factors, heavy metal pollution poses serious threats to the environment and human health. Several studies have focused on benefits of symbioses for the host plant growing on contaminated sites. Ecto- and endomycorrhizal symbioses can play a crucial role in protecting plants from toxic metals (Meharg and Cairney, 2000; Meharg, 2003). The ability of mycorrhizal associations to ameliorate metal toxicity to higher plants has been shown for ericoid mycorrhizas (Bradley *et al.*, 1981), ectomycorrhizas (Adriaensen *et al*., 2003; Colpaert *et al.*, 2004) and arbuscular mycorrhizas (Gildon and Tinker, 1983; Heggo and Angle, 1990). However, the targets of toxicity are not fully known for most heavy metals, as well as the cellular systems contributing to tolerance acquisition. Insertional mutagenesis is a powerful tool to study the function of a gene and the biology of an organism, and it could represent an effective way to study mechanisms of heavy metal and, more in general, oxidative stress tolerance. Much of the current knowledge on the genetic transformation of mycorrhizal fungi derives from studies on ectomycorrhizal fungi such as *Laccaria laccata* (Barrett *et al.*, 1990), *Hebeloma cylindrosporum* (Marmeisse *et al.*, 1992; Pardo *et al.*, 2002; Combier *et al.*, 2003), *Paxilus involutus* (Bills *et al.*, 1995; Pardo *et al.*, 2002), *Laccaria bicolor* (Bills *et al.*, 1999; Kemppainen *et al.*, 2005; Kemppainen *et al.*, 2009), *Suillus bovinus* (Hanif *et al.*, 2002; Pardo *et al.*, 2002), and *Pisolithus tinctorius* (Rodriguez-Tovar *et al.*, 2005). The only report on the stable genetic transformation of an endomycorrhizal fungus was the transformation of the ericoid ascomycete *Oidiodendron maius* strain Zn (Martino *et al.*, 2007; Abbà *et al.*, 2009).

Oidiodendron maius Zn is an interesting organism because, in addition to being a mycorrhizal fungus, it is also a heavy metal-tolerant strain (Martino *et al.*, 2000). However, the mechanisms that allow this fungus to survive and to tolerate toxic concentrations of heavy metals are poorly understood. As a useful tool to search for genes involved in the tolerance mechanisms, a collection of random-mutants was prepared for this fungus (Abbà, unpublished) and partially screened on zinc, cadmium and menadione.

Once a phenotype of interest is identified, the main task is to isolate the DNA sequences flanking the hygromycin cassette used for random insertion. Several molecular methods have been proposed for the recovery of DNA sequences adjacent to known fragments, such as inverse PCR (Ochman *et al.*, 1988; Triglia *et al.*, 1988), adapter-ligation-mediated PCR

(Mueller and Wold, 1989; Jones and Winistorfer, 1992), and thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier, 1995; Liu *et al.*, 1995).

In the present study, the TAIL-PCR method was used for the identification of the disrupted genes. This method (Fig. 1) makes use of three nested sequence-specific primers designed on the hygromycin cassette and an arbitrary degenerate primer having a low melting temperature, so that the relative amplification efficiencies of specific and non-specific products can be thermally controlled (Liu and Whittier, 1995; Liu et *al.*, 1995). In the primary reaction, one low-stringency PCR cycle is conducted to create one or more annealing sites for the arbitrary degenerate primer along the targeted sequence. Specific products are then preferentially amplified over nonspecific ones by interspersion of two high-stringency PCR cycles with one reduced-stringency PCR cycle. The nested PCR amplifications help to achieve higher specificity (Liu and Huang, 1998).

Here we report the different steps and the protocol used for the screening of the random-mutants collection, and the identification of three mutated genes.

2. Materials and Methods

2.1. Optimisation of the culture medium for mycelium growth

As the effects of heavy metals on fungal growth also depend on the type of culture medium, trials were carried out to find the optimal medium for *O. maius* growth. The effects of increased heavy metal concentrations on morphological parameters such as radial growth and the general mycelium aspect (e.g. colour, pigmentation, production of exudates) were taken into consideration. The wild type (WT) strain was grown on three different solid culture media: [1] Czapek-glucose (2%) containing $NaNO_3$ (3 g l^{-1}), $K_2HPO_4.3H_2O$ (1.31 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}), KCl (0.5 g l^{-1}), $FeSO_4.7H_2O$ (0.01 g l^{-1}); [2] Czapek-Dox containing $NaNO_3$ (2 g l^{-1}), K_2SO_4 (1.31 g l^{-1}), $MgC_3H_9O_6P$ (0.5 g l^{-1}), $FeSO_4.7H_2O$ (0.01 g l^{-1}), KCl (0.5 g l^{-1}) and sucrose (30 g l^{-1}); [3] *Aspergillus* Complete Medium (ACM) containing $NaNO_3$ (6 g l^{-1}), $MgSO_4.7H_2O$ (0.52 g l^{-1}), KCl (0.52 g l^{-1}), K_2HPO_4 (1.52 g l^{-1}), $FeSO_4.7H_2O$ (0.01 g l^{-1}), $ZnSO_4.7H_2O$ (0.01 g l^{-1}), casamino acids (1.5 g l^{-1}), yeast extract (1.5 g l^{-1}), bacteriological peptone (2 g l^{-1}) and glucose (2 g l^{-1}). All media were solidified with 1% agar, adjusted to pH 6 (except ACM medium maintained at the initial pH of 5.52) and autoclaved. Zn^{2+} was added as $ZnSO_4.7H_2O$ (Fluka) to reach a final concentration of 5, 10, 15, 20 and 25 mM. Cd^{2+} was added as $3CdSO_4.8H_2O$ (Sigma) to reach a final concentration of 0.01, 0.05, 0.1, 0.15 and 0.2 mM. Petri dishes were kept in the dark at 25°C, and radial growth was recorded using a ruler at regular weekly intervals over 8 weeks. At least three replicates were performed for each experimental condition.

2.2. Toxic substance concentrations

O. maius WT was used to determine the concentration threshold of Zn, Cd and menadione causing at least a 50% reduction in the mycelium radial growth as compared to the control. The fungus was grown on Czapek-glucose (2%) medium adjusted to pH 6 and supplemented with 1% agar. Substance concentration ranges were 5-100 mM for zinc, 0.01-0.9 mM for cadmium and 0.1-40 mM for menadione. Petri dishes were kept in the dark at 25°C, and the diameters of the fungal mycelium were recorded using a ruler at regular weekly intervals over 8 weeks. At least three replicates were prepared for each experimental condition.

2.3. Mutant screening

The random mutants were obtained by *Agrobacterium*-mediated transformation using either the pUR5750 (de Groot *et al*., 1998) or the pCAMBIA0380 (Cambia, Canberra, Australia) plasmid (Abbà, unpublished). These mutants were firstly inoculated on 5 cm-petri dishes, then

transferred on 9 cm-plates containing Czapek-glucose medium supplemented with 15 mM Zn, 0.3 mM Cd or 0.75 mM menadione. Radial growth was recorded at regular weekly intervals over 4 weeks. The sensitive mutants were also grown for 30 days in liquid Czapek medium supplemented with Zn, Cd, and menadione to determine the mycelium biomass. At least three replicates were performed for each experimental condition. This screening lead to the selection of mutants that show a reduction of mycelium growth on at least one of the three toxic substances. Selected candidates have been further analysed for the identification of the mutated genes.

2.4. Southern blot experiments

Ten micrograms of genomic DNA from the WT and from the selected mutants were extracted using DNeasy plant mini-kit (Qiagen) following manufacturer instructions. Genomic DNAs were than digested with BglII and size-fractionated on a 1% (w/v) agarose gel.The separated restriction fragments were blotted onto nylon membranes following standard procedures (Sambrook and Russell, 2001). Hybridisation with a chemiluminescent detection system (ECL Direct DNA labelling and Detection System, Amersham) was performed according to the manufacturer's recommendations. A probe specific for the hygromycin phospho-transferase gene was used to verify the number of plasmid insertions in the mutant genome. The probe corresponded to a purified 321 bp sequence spanning the hph gene obtained by PCR amplification with the primers HygF (ATGCCTGAACTCACCGCGAC) – HygR (GCAGTTCGGTTTCAGGCAGG). Probe labelling and high stringency hybridisation were carried out using the ECL protocol (Amersham Pharmacia).

2.5. Identification of the disrupted genes

Thermal Asymmetric Interlaced (TAIL)-PCR method was used for the identification of genes involved in heavy metal tolerance. This method is considered to be a powerful tool for the recovery of DNA fragments adjacent to known sequences. The two arbitrary degenerate primers AD1 and AD2 (Liu and Whittier, 1995) were used in combination with specific primers designed on the nucleotide sequence of the pCAMBIA0380 or pUR5750 vectors (Table 1 and Fig. 1) used for the transformation. The primary reaction (30 µl) contained 6 µl PCR-buffer (5X); 200 µM of dNTPs; 0.5 µM of a specific primer, 0.5 µM of an arbitrary degenerate (AD) primer, 0.3 µl of *Taq* DNA polymerase (Phusion) and 30 ng/µl of DNA. For the secondary reaction (20 µl), 1 µl of the 50-fold diluted primary reaction product was added to the PCR mixture containing 4 µl of PCR-buffer (5X); 100 µM of dNTPs; 0.5 µM of internal specific primer, 0.5 µM of the same AD primer and 0.2 µl of *Taq* DNA polymerase

(Phusion). A final nested PCR was performed using 1 µl of the 10-fold diluted secondary reaction product. Cycling conditions were performed with a modification of those described previously by Liu and Whittier (1995) (Table 2). The amplified fragments were cloned into the pCR2.1-TOPO vector (TOPO TA cloning, Invitrogen, and the recombinant plasmids were transformed into TOP10-*E. coli* (Invitrogen) using the electroporation method. Positive clones were sequenced and resulting sequences were blasted to identify similarities to known proteins.

Table 1. Primers used for the TAIL-PCR method

Feature	Plasmid	Primer name	Primer sequence (5'-3')
Arbitrary degenerate primers	pCAMBIA0380 or pUR5750	AD1	GTNCGASWCANAWGTT
		AD2	WGTGNAGWANCANAGA
Nested specific primers used in the upstream TAIL-PCR	pCAMBIA0380	Cam5P1	CCTGTTGCCGGTCTTGCGATGA
		Cam5P2	GCATGACGTTATTTATGAGATGGG
		Cam5P3	TAGATCGGGAATTAAACTATCAGTG
	pUR5750	pUR5P1	TCGCCCTTTGACGTTGGAG
		pUR5P2	CAACACTCAACCCTATCTCC
		pUR5P3	CGGAACCACCATCAAACAGG
Nested specific primers used in the downstream TAIL-PCR	pCAMBIA0380	Cam3P1	CATCATCAAGGCACCCCG
		Cam3P2	CCTATGCCCACCGCCGATCAG
		Cam3P3	GCCTCCTCTAAGCTTGGCTG
	pUR5750	pUR3P1	GCATCAGAGCAGCCGATTGT
		pUR3P2	ATAGCCTCTCCACCCAAGCG
		pUR3P3	GGAATTTATGGAACGTCAGTGG

Table 2. Cycling conditions used for TAIL-PCR

Primary reaction			**Secondary reaction**			**Tertiary reaction**		
Step	**Temperature (°C)**	**Time (min:s)**	**Step**	**Temperature (°C)**	**Time (min:s)**	**Step**	**Temperature (°C)**	**Time (min:s)**
1	92	2:00	1	98	0:30	1	98	0:30
2	98	0:30	2	98	0:10	2	98	0:10
3	98	0:10	3	63	1:00	3	44	1:00
4	63	1:00	4	72	2:00	4	72	2:00
5	72	2:00	5	98	0:10	5	Go to step 2	35 times
6	Go to step 3	5 times	6	63	1:00	6	72	5:00
7	98	0:10	7	72	2:00	7	End	
8	30	3:00	8	98	0 :10	8		
9	72	2:00	9	44	1:00	9		
10	98	0:10	10	72	2:00	10		
11	44	1:00	11	Go to step 2	10 times	11		
12	72	2:00	12	72	5:00	12		
13	Go to step 10	10 times	13	End				
14	98	0:10						
15	63	1:00						
16	72	2:00						
17	98	0:10						
18	63	1:00						
19	72	2:00						
20	98	0:10						
21	44	1:00						
22	72	2:00						
23	Go to step 14	12 times						
24	72	5:00						
25	End							

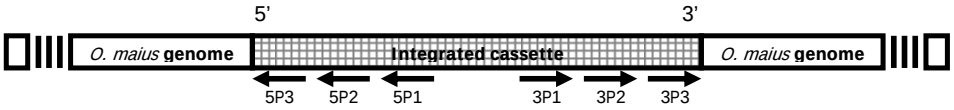

Figure 1. Orientation of the specific primers used in the TAIL-PCR method

2.6. Statistical analyses

Statistical analyses were performed through one-way ANOVA and Tukey's post hoc test, using a probability level of $P < 0.05$.

3. Results

3.1. Choice of the growth medium for the mutant screening and toxic substance concentrations

O. maius strain Zn was grown on three different solid culture media: Czapek-glucose (2%), Czapek-Dox (3%) and ACM medium. Among the three media tested, Czapek-glucose was chosen for the subsequent screening experiments because it allowed rapid mycelial growth and was the best medium to highlight the growth differences on the different metal concentrations (Fig. 2). The concentration of the toxic substances causing a 50% reduction in the WT mycelial growth was 15 mM for Zn, 0.3 mM for Cd, and 0.75 mM for menadione (data not shown).

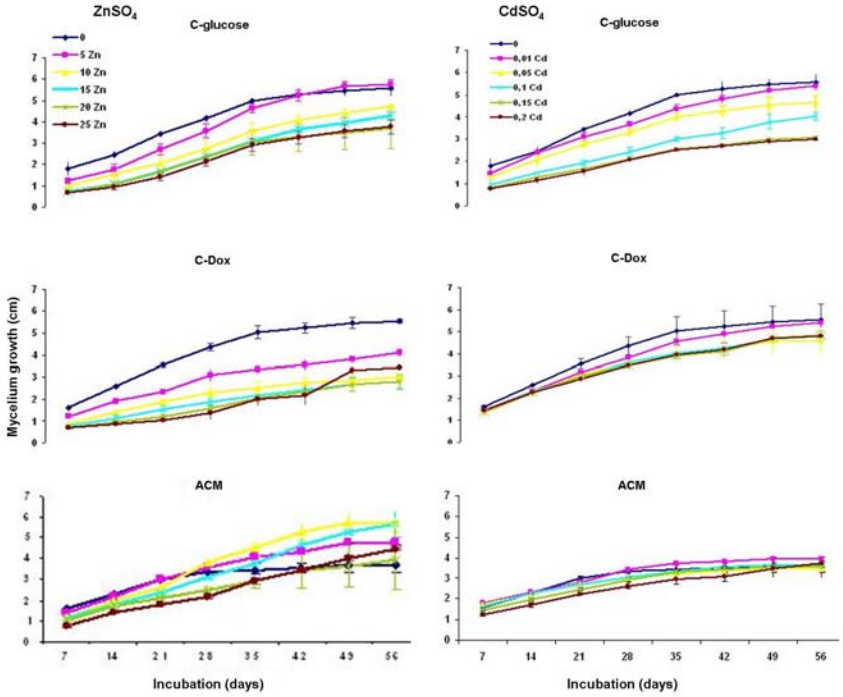

Figure 2. Growth of *O. maius* Zn mycelium on Czapek-glucose, Czapek-Dox and *Aspergillus* Complete Medium (ACM) media supplemented with increasing concentrations (mM) of $ZnSO_4$ (left panels) and $CdSO_4$ (right panels).

3.2. Mutants screening and molecular analyses

From the collection of *O. maius* random-mutants, 309 were further tested on plates containing Czapek-glucose medium supplemented with 15 mM Zn, 0.3 mM Cd and 0.75 mM menadione. When compared to the wild type, 23 mutants (about 7%) showed a significant reduction of mycelial growth on at least one of the three toxic substances (Fig. 3). Ten out of the 23 candidates revealed sensitivity to all three substances. Twenty mutants (6.47 %) were

zinc sensitive, 14 mutants (4.53 %) were Cd sensitive and 13 mutants (4.2 %) were menadione sensitive (Fig. 3). In some cases, growth was completely inhibited, as found for *O. maius* A, a heavy metal sensitive strain isolated from a non polluted site (Martino *et al.*, 2000) (Fig. 4). Southern blot hybridization for these 10 mutants revealed that only one copy of the transformation cassette was integrated in the genome (data not shown).

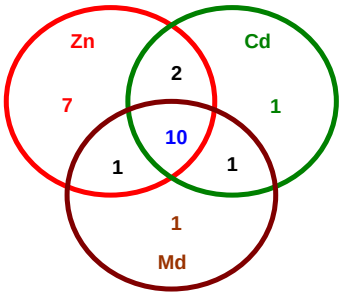

Figure 3. Venn diagram showing the distribution of the 23 fungal mutants according to their sensitivity to zinc (Zn), cadmium (Cd) and menadione (Md)

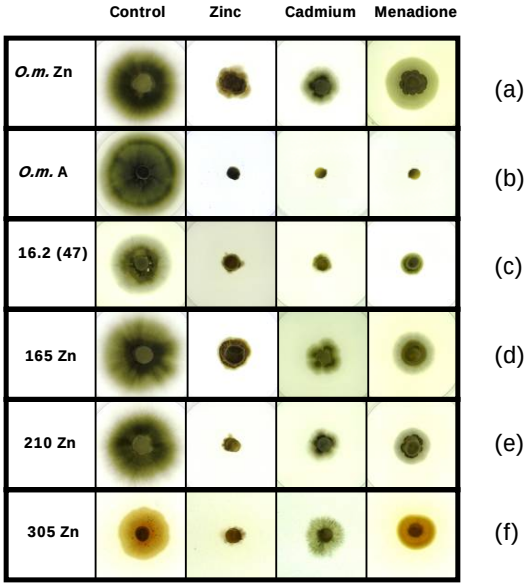

Figure 4. Mycelial growth of O. maius Zn wild type (a), *O. maius* A (a heavy metal sensitive strain) (b), and some random mutants (c-f) that showed a significant reduction in mycelial growth on at least one of the three toxic substances tested. Strains were grown on Czapek-glucose medium supplemented with 15 mM $ZnSO_4$; 0.3 mM $CdSO_4$ and 0.75 mM menadione.

3.3. Identification of the mutated genes

Although the identification of the insertion sites by TAIL-PCR is still in progress, three mutated genes have been already identified and are listed below.

- *OmMFS*: Major Facilitator Superfamily

This mutated gene was named *OmMFS* as it shared 63% and 60% amino acid identity with the MFS transporter of *N. crassa* and *A. fumigatus*, respectively. The mutant and the WT showed similar radial growth on the control medium, whereas growth of this mutant was reduced, as compared to the WT, when exposed to menadione. The mutant and the WT did not show any growth difference on the medium supplemented with zinc and cadmium (Fig. 4d).

- *Om11.1*: bZIP transcription factor

This mutant was altered in a basic leucine zipper (bZIP) transcription factor. In the control medium, the biomass of the mutant was reduced by 41% with respect to the WT (Fig. 5). In the presence of Zn (Fig. 5), Cd and menadione (data not shown), the difference between the WT and the *Om11.1* mutant became more pronounced.

- *OmNmr1*: nitrogen metabolite repression gene

This gene shared 45% and 44% amino acid identity with the Nmr repressors *nmr-1* of *N. crassa* and *nmrA* of *A. nidulans*, respectively. On the control liquid medium, no significant differences in dry biomass were recorded between the *OmNmr1* mutant and the WT (Fig. 6). By contrast, in the presence of toxic substances, the mutant biomass was significantly lower when compared to that of the WT. The most pronounced reduction was observed when mycelia were exposed to zinc (Fig. 6).

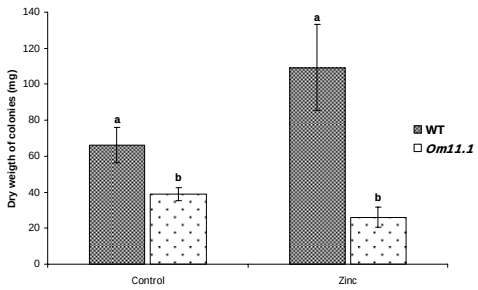

Figure 5. Dry weigth of the WT and the *Om11.1* mutant grown on either control liquid medium or on medium supplemented with 15 mM zinc.

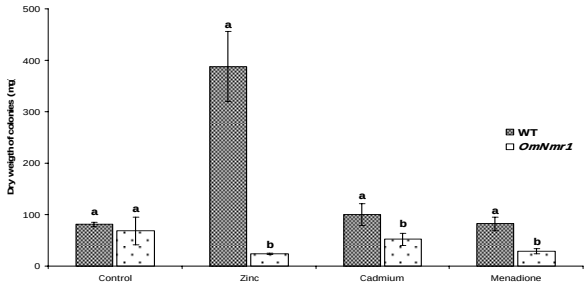

Figure 6. Dry weigth of the WT and the *OmNmr1* mutant grown on either control liquid medium or on medium supplemented with 15 mM zinc, 0.3 mM cadmium, or 0.75 mM menadione.

Each column with the same letters are statistically not different according to Tukey's post hoc test, using a probability level of $P < 0.05$.

4. Discussion

An effective way to investigate the mechanisms of stress tolerance in fungi is to disrupt the genes involved in order to generate mutants exhibiting altered tolerance, either by a targeted or by a random process. Although a number of techniques can be employed for such an analysis, those based on transformation have proved to be highly effective. In filamentous fungi, the introduction of DNA by transformation typically results in either heterologous or homologous integration of the transforming DNA into the target genome. Homologous integration leads to the knock-out of a targeted gene by disruption of the wild-type allele with the transformation cassette. By contrast, heterologous integration of transforming DNA causes a random process of gene disruption (random insertional mutagenesis). Both homologous and heterologous integration have been widely used to study fungal processes. For example, they have been used to determine the role in pathogenicity of newly isolated fungal genes and to isolate fungal mutants defective in pathogenicity, respectively (Mullins and Kang, 2001). The main advantage of random insertional mutagenesis, over the more traditional chemical or radiation mutagenesis procedures, is that the mutated gene is tagged by the nucleotide sequence of the vector used for transformation. A random-mutant collection is therefore a powerful tool to screen for a phenotype of interest and to characterize the function of a gene.

Our main aim was the identification of genes involved in heavy metal and/or oxidative stress tolerance in a metal-tolerant ericoid mycorrhizal fungus, *O. maius*. We generated and screened a collection of random-mutants to search for mutants impaired in their growth ability on at least one of the three toxic substances tested (zinc, cadmium and menadione). Out of the 309 mutants screened, 7% proved to be sensitive to at least one of the three substances used and 3% was sensitive to all three toxic compounds. More in detail 20 mutants (6.47 %) were zinc sensitive, 14 mutants (4.53 %) were Cd sensitive and 13 mutants (4.2 %) were menadione sensitive. Pagani *et al*. (2007), with the aim to evaluate cellular functions required to survive and proliferate under a surplus of zinc cations, screened a library of 4825 *S. cerevisiae* haploid deletion mutants on zinc. They found that 1.8% of the mutants (89 out of 4825) showed an increased sensitivity to high concentrations of zinc in the medium. Ruotolo *et al.* (2008) examined the fitness of a genome-wide collection of yeast deletion mutant strains exposed to two chemically diverse metals, namely cadmium and nickel. They found that 303 (6.4%) out of 4688 total mutants were Cd-sensitive and 118 (2.5%) were Ni-sensitive.

Using the TAIL-PCR technique, we were able to identify the mutated genes for three of the identified mutants.

The first gene identified was a member of the major facilitator superfamily (MFS). MFS proteins facilitate the transport across the plasma membrane or internal membranes of a variety of substrates including ions, sugar phosphates, drugs, amino acids, and peptides (Pao *et al.*, 1998; Walmsley *et al.*, 1998). This gene could play an important role in metal tolerance, as previously reported for a number of filamentous fungi (Hayashi *et al.*, 2002; Morschhäuser, 2010), yeast (Panwar *et al.*, 2008), and plants (Haydon and Cobbett, 2007; Cabrito *et al.*, 2009). MFS transporters may in fact prevent accumulation of toxic compounds in cells, and their activity is driven by the proton-motive force over membranes (Pao *et al.*, 1998).

The second gene identified is a putative basic leucine zipper (bZIP) transcription factor. bZIP transcription factors are found in fungi, animals, plants and viruses (Shelest, 2008). In fungi, bZIPs have been proposed to be involved in the regulation of a broad spectrum of stress responses and in the process of mycelial growth. In our case, even in the absence of an oxidative stress, the mycelial growth of this particular mutant was significantly reduced when compared to the WT. These findings are in accordance with the important role of the bZIP transcription factors as regulators of cellular differentiation and growth (Guo *et al.*, 2010).

Finally, the third gene identified is the *OmNmr1* gene, which is reported to be a negative regulator in nitrogen metabolism and is highly conserved among all filamentous fungi (Wong *et al.*, 2008). Further investigation of the *OmNmr1* mutant phenotype will be the subject of the next chapter.

Screening of large collections of random-mutants are reported in literature for different organisms and for different purposes. Hill *et al*. (2006) screened a collection of *Aspergillus nidulans* mutants to isolate cell wall mutants by screening for hypersensitivity toward the chitin binding agent Calcofluor White. This strategy was used previously to identify cell wall mutants in *Saccharomyces cerevisiae*. These authors identified 10 Calcofluor hypersensitive mutants. Seong *et al*. (2005), used the restriction enzyme-mediated integration (REMI) approach to generate random insertional mutants with the aim to understand the pathogenesis molecular mechanisms in the wheat scab fungus *Fusarium graminearum*. Eleven pathogenicity mutants were identified by screening of 6,500 hygromycin-resistant transformants. Marmeisse *et al*. (2004) studied the basidiomycete *Hebeloma cylindrosporum*. Combined biochemical, cytological, genetical and molecular approaches led to the characterisation of mutant strains affected in mycorrhiza formation. Dias *et al*. (2010) used *Saccharomyces cerevisiae* to uncover the mechanisms underlying tolerance and toxicity of the agricultural fungicide mancozeb. Thanks to a chemogenomics screening of a collection of yeast deletion mutants, they were able to identify 286 genes that provided protection against

mancozeb toxicity. Roemer *et al.* (2003) constructed a conditional mutant strain collection with the aim to select preferred drug targets from the *Candida albicans* essential gene set. In this way, they were able to identify 567 essential genes in *C. albicans*. Thorsen *et al.* (2009) carried out a genome-wide screening of a collection of *S. cerevisiae* deletion mutants and scored for reduced growth in the presence of arsenite or cadmium, to gain insight into metal action and cellular tolerance mechanisms. In this way, these authors were able to elicite some of the processes required for tolerance to both metals, among them sulphur and glutathione biosynthesis, environmental sensing, mRNA synthesis and transcription, and vacuolar/endosomal transport and sorting.

Pagani *et al.* (2007), in a genomewide transcriptional profiling by screening a library of *S. cerevisiae* haploid deletion mutants on zinc (as reported above), revealed that exposure to 5 mM $ZnCl_2$ resulted in rapid increase in the expression of numerous chaperones required for proper protein folding or targeting to vacuole and mitochondria, as well as genes involved in stress response (mainly oxidative), sulphur metabolism and some components of the iron regulon. Ruotolo *et al.* 2008 used a genomic phenotyping, by screening a collection of yeast deletion mutant strains (as reported above), to assess the roles played by all nonessential *S. cerevisiae* proteins in modulating cell viability after exposure to cadmium, nickel, and other metals. A number of novel genes and pathways that affect multimetal as well as metal-specific tolerance were discovered. Although confirming the high importance of the vacuole in the metal detoxification mechanism, they also identified a number of pathways that play a more general, less direct role in promoting cell survival under stress conditions (for example, mRNA decay, nucleocytoplasmic transport, and iron acquisition) as well as proteins that are more proximally related to metal damage prevention or repair. Very interesting among the latter are various nutrient transporters previously not associated with metal toxicity.

A number of papers also reported the screening of large collection of plant mutants to isolate mutants with altered sensitivity to cadmium (Watanabe *et al*., 2010; Tsyganov *et al*., 2007), iron (Cheng *et al*., 2007) and aluminium (Kikui *et al*., 2005; Ma *et al*., 2005; Nawrot *et al.* (2001). David-Schwartz *et al.* (2003) isolated a pre-mycorrhizal infection (pmi2) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonization through a direct screening for Myc(-) plant mutants.

Thus, the screening of large collections of random-mutants has been highly applied to yeasts, filamentous fungi and plants, considering different stressfull situations, cell cycle stages or pathogenesis/symbiosis events. Anyway, to our knowledge, it was never applied to filamentous fungi with the purpose to study heavy metal and oxidative stress tolerance. This work, although the mutant collection screening has to be continued, represents therefore the

first attempt to use this technique for this kind of analyses and for the identification of mutated genes in mycorrhizal fungi.

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Chapter 5

***OmNmr1* mutation in the ericoid mycorrhizal fungus *Oidiodendron maius* induces a reorganization of the N pathway**

Abstract

A random-mutant collection of *Oidiodendron maius* strain Zn, a metal tolerant ericoid mycorrhizal fungus, was screened for sensitivity to oxidative stress causing substances. Among highly sensitive mutants, one mutant was chosen for further analyses. The mutated gene identified by TAIL-PCR, is involved in the nitrogen metabolite repression mechanism, and is regulated in response to nitrogen availability and quality. The mutant, further named *OmNmr1*, showed an increased sensitivity to chlorate and thiourea, suggesting the inactivation of this gene. To characterize the phenotype of the *OmNmr1* mutant, the amino acid pools were evaluated for both the WT and the mutant using GC-MS, and the activity of key enzymes was measured. The mutant was characterized by an increase of glutamate and alanine concentrations and a decrease of glutamine and asparagine amounts. These amino acid patterns were correlated with a strong decrease of the glutamine synthetase (GS) and a concomitant increase of the glutamate dehydrogenase (GDH) activity. The reduction of the GS activity could explain the reduced amount of glutamine and asparagine measured. An increase of the mutant mycelium biomass on glutamine containing medium, compared to the other nitrogen sources, was measured. Possible links between the oxidative stress tolerance and the nitrogen metabolism are discussed.

1. Introduction

Filamentous fungi can use a wide variety of compounds as nitrogen sources. Ammonium, glutamine and glutamate are readily assimilated by fungi and are preferentially used, but when these nitrogenous compounds are lacking, other secondary sources can be used (Marzluf, 1997). In response to the nitrogen status of the cell, complex mechanisms are involved in the control of nitrogen utilization and in the preferentially use of nitrogen compounds. Two ascomycetes, *Aspergillus nidulans* and *Neurospora crassa*, have been widely used as models to study the regulation of nitrogen source utilization, a process termed nitrogen metabolite repression (NMR). This mechanism is activated by regulatory proteins containing a DNA binding domain consisting of a Cys-2/Cys-2 zinc-finger motif characteristic of the GATA-type transcription factors (Scazzocchio, 2000). The two transcription factors *areA* (Kudla *et al.*, 1990; Langdon *et al.*, 1995) and *nit-2* (Stewart and Vollmer, 1986; Fu and Marzluf, 1990) from *A. nidulans* and *N. crassa*, respectively, are reported to be involved in NMR. Loss-of-function mutants *areA* and *nit-2* are unable to use non-preferred nitrogen sources. Under nitrogen sufficient conditions, the activity of AreA and Nit2 is inhibited by binding to the negatively acting regulator NmrA (Andrianopoulos *et al*., 1998) and Nmr-1 (Fu *et al.*, 1988), respectively, which are highly conserved among all filamentous fungi (Wong *et al.*, 2008). Consequently, deletion of *nmr* results in partial derepression of nitrogen-regulated genes. However, overexpression of *nmr* leads to partial repression of AreA activity (Wagner *et al.*, 2010).

Most fungi possess two metabolic pathways involved in the assimilation of ammonium and the biosynthesis of glutamate (Marzluf, 1997; Inokuchi *et al.*, 2002). One route involved the NADP-linked glutamate dehydrogenase (NADP-GDH), which catalyses the reversible amination of 2-oxoglutarate to form glutamate (Botton and Dell, 1994). The second route involved the combined action of glutamine synthetase (GS), which aminates glutamate to form glutamine, and glutamate synthase (GOGAT), which transfers the amino group of glutamine to 2-oxoglutarate resulting in the production of two molecules of glutamate. Glutamate is an essential amino nitrogen donor for many transaminases and glutamine is the precursor of many essential metabolites such as nucleic acids, amino sugars, asparagine, histidine and tyrosine (Javelle *et al*., 2004).

Mycorrhizal fungi play an important role in plant nutrition and plant health (Smith and Read, 1997). The success of ericaceous plants to grow in acidic soils with low availability of mineral nitrogen and phosphorus is attributed to their symbiotic association with ericoid fungi (Cairney and Meharg, 2003; Read and Perez-Moreno, 2003). The generation of fungal

mutants altered in nitrogen regulatory genes could be a powerful tool to investigate the nitrogen metabolism, and further, to characterize some features of the mycorrhizal symbiosis and the improvement of nitrogen nutrition in the host plant. *Oidiodendron maius* Zn, an ascomycete belonging to the class Leotiomycetes, can establish an endomycorrhizal symbiosis with the roots of ericaceous plants (Perotto *et al*., 1996; Hambleton *et al.*, 1998; Chambers *et al.*, 2000) and was previously characterized for its elevated metal tolerance (Martino *et al*., 2000; Martino *et al.*, 2002; Martino *et al*., 2003).

In this work, we report the generation and characterization of a *O. maius* Zn random mutant, hereafter referred to as *OmNmr1* mutant. The *OmNmr1* mutant was identified in a screening for oxidative stress tolerance of an *O. maius* mutant collection obtained through random insertional mutagenesis (Abbà, unpublished).

2. Materials and Methods

2.1. Fungal isolate and growth media

The fungal isolate investigated in this work is *Oidiodendron maius* Zn. This strain was isolated from the roots of *Vaccinium myrtillus* plants grown in the Niepolomice Forest (25 km northeast of Krakow, Poland). This experimental area was treated with 5000 tons/km^2 of dust containing high concentrations of zinc, cadmium and aluminium (Martino *et al.*, 2000). Fungi were grown in Czapek-glucose mineral medium containing 35 mM $NaNO_3$, 5.7 mM $K_2HPO_4 \cdot 3H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 6.7 mM KCl, 36 µM $FeSO_4 \cdot 7H_2O$ and 2% (w/v) glucose (Sigma, St. Louis). Media were buffered at pH 4 using 20 mM MES (2-[N-morpholino] ethane sulphonic acid) and HCl 1 M before autoclaving.

2.2. Mutant screening

A mutant collection of the strain *O. maius* Zn was previously obtained through random insertional mutagenesis (Abbà, unpublished). These mutants were obtained by *Agrobacterium*-mediated transformation using either the pUR5750 (de Groot *et al.*, 1998) or the pCAMBIA0380 (Cambia, Canberra, Australia) plasmid. The mutants were screened for their ability to grow on 15 mM $ZnSO_4$, 0.3 mM $CdSO_4$ and 0.75 mM menadione in respect to the wild-type strain (WT).

2.3. Southern blot

The number of insertions in the genome was checked by the southern hybridization analysis. The genomic DNA of the mutant and the WT was extracted from mycelium grown for 30 days in liquid Czapek-glucose medium using DNeasy plant mini-kit (Qiagen) following manufacturer instructions. Genomic DNAs were than digested with *BglII* restriction enzyme and were size-fractionated on a 1% (w/v) agarose gel. The blot was probed with a fragment corresponding to a purified 321 bp sequence spanning the *hph* gene obtained by PCR amplification with the primers HygF (ATGCCTGAACTCACCGCGAC) – HygR (GCAGTTCGGTTTCAGGCAGG). Probe labelling and hybridization were performed with a chemiluminescent detection system (ECL direct DNA labeling and detection system; GE Healthcare, Chalfont St. Giles, U.K.) following the manufacturer's protocol.

2.4. Identification of the mutated gene

The identification of the mutated gene was performed using the Thermal Asymmetric Interlaced PCR (TAIL-PCR) method. This method is reported to be a powerful tool for the

recovery of DNA fragments adjacent to known sequences (Liu and Huang, 1998). The two arbitrary degenerate primers AD1 and AD2 (Liu and Whittier, 1995) were used in combination with specific primers designed on the nucleotide sequence of the pCAMBIA0380 vector, which was used for the transformation (see chapter 4-Materials and Methods). Cycling conditions for TAIL-PCR were performed with a modification of those described previously by Liu and Whittier (1995) (see chapter 4-Materials and Methods).

2.5. Growth assays on $KClO_4$ and Thiourea

Several tests have been previously reported to determine the derepression of activities subjected to nitrogen metabolite repression (Platt *et al.*, 1996; Andrianopoulos *et al*., 1998; Schönig *et al*., 2008; Jiang *et al.*, 2009). The WT and the *OmNmr1* mutant were grown for 30 days on solid Czapek-glucose medium, in the presence of ammonium tartrate as a nitrogen source instead of $NaNO_3$, and supplemented with 125 mM potassium perchlorate ($KClO_4$) or 4 mM thiourea.

2.6. Amino acid extraction and analysis

Both the WT and the *OmNmr1* mutant were grown for 30 days in liquid Czapek-glucose medium with NO_3 as a nitrogen source and starved from nitrogen for 1 day. Nitrate was then re-supplied to the medium at a 35 mM concentration and mycelia were harvested after 16h, 24h and 48h and immediately frozen in liquid nitrogen. Amino acids were extracted twice from freeze-dried mycelium with 2 x 300 µl of 70% (v/v) cold ethanol. Extracts were dried under N_2 using a Reacti-Therm Heating Module (Pierce, Rockfort, IL, USA) and resuspended in 400 µl of HCl (0.12N). Extracts and standards were loaded onto a Dowex 50WX-8 cation ion exchange column (Sigma-Aldrich, St Louis, MO, USA) with the addition of 20 µl of norleucine for the calibration. Samples were washed twice with 1 ml of sterile water, and amino acids were eluted with 4 ml (2ml for 2 times) of 4.5N ammonia. Derivatization and gas chromatography-mass spectrometry (GC-MS) analyses were performed according to Javelle *et al.* (2003).

2.7. Enzyme and protein assays

Wild type and mutant strains were grown on solid Czapek-glucose medium containing 10 g/l of agar and supplemented with 0.49 g/l of the following N sources: sodium nitrate, ammonium sulfate, ammonium tartrate, glutamine, or glutamate, at pH 5.6. Prior to inoculation, sterile cellophane membranes were placed aseptically on the agar surface to provide a convenient means of removing the mycelium from the plate. The membranes were

prepared by first boiling for 15 min in 10 mM EDTA, rinsing and then autoclaving in dH_2O. Fungal colonies (3 replicates/treatment) were removed after 30 days by peeling the biomass from the cellophane membranes. Mycelia were then frozen at -80°C. Enzymatic assays were performed according to Brun *et al.* (1992). Proteins were extracted from about 200 mg of mycelium, with the addition of 10% (w/w) of polyvinylpolypyrrolidone and 1 ml of a cold extraction buffer consisting of 50 mM Tris-HCl (pH 7.6); 5 mM $MgSO_4$; 2 mM Na_2EDTA; 20% (v/v) glycerol; 2% (w/v) polyvinylpyrolidone and 14 mM 2-mercaptoethanol. Extracts were centrifuged at 14000 rpm for 15 min at 4°C, and the supernatants were used for the determination of protein amounts and of GS and GDH activities. Soluble protein content was assayed by a modification of the Bradford method (1976). The absorbance was measured at 595 nm. GS activity was determined by a modification of the transferase assay of Shapiro and Stadtman (1970). The reaction mixture contained 10 mM Tris-HCl (pH 7.2), 125 mM L-glutamine, 30 mM NH_2OH, 20 mM KH_2AsO_4, 4 mM EDTA, 20 mM $MgSO_4$, 0.5 mM ADP and 200 µl of the enzymatic extract. The absorbance was measured at 540 nm. NADP-GDH activity was performed by following NADPH oxidation at 340 nm. The reaction mixture was composed of 100 mM potassium phosphate buffer, 122 mM ammonium phosphate, 8.7 mM 2-oxoglutarate, 156 µM NADPH and 100-150 µl of the enzymatic extract.

2.8. Mycorrhizal synthesis

In vitro endomycorrhizae were synthesized as described by Abbà *et al.* (2009) with some modifications. Ten axenic *V. myrtillus* seedlings, obtained from seeds coming from Les Semences du Puy, Le Puy-En-Velay, France, were inoculated on modified Ingestad's medium (Ingestad, 1971), where four mycelium plugs of both the WT and the mutant were previously grown for 45 days. Plates were placed in a growth chamber (16h photoperiod, light at 170 µmol m^{-2} s^{-1}, temperatures at 23°C day and 21°C night), and roots were observed after 3 months of incubation.

3. Results

3.1. Mutant screening

A random mutant collection of *O. maius* strain Zn was screened for sensitivity to 15 mM Zn, 0.3 mM Cd and 0.75 mM menadione in respect to the WT. Among sensitive mutants, a mutant showing high sensitivity to all the tested substances was selected. A slight reduction of the radial growth was observed in the mutant in respect to the WT on control medium (Fig. 1). By contrast, in the presence of oxidative stress causing substances the mycelial growth of the mutant was remarkably reduced, or even inhibited, as compared to the WT (Fig. 1).

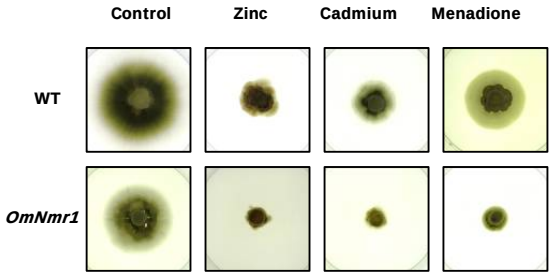

Figure 1. Determination of oxidative stress tolerance of *OmNmr1*-mutant. The WT and the mutant were grown on agar medium supplemented or not (control) with 15 mM $ZnSO_4$, 0.3 mM $CdSO_4$ and 0.75 mM of menadione. Pictures were taken after 30 days of growth.

3.2. *OmNmr1* encodes a transcription factor involved in the repressor of nitrogen utilization

To check the number of integrations of the hygromycin cassette, the blot was probed with a fragment of the hygromycin resistance gene. Figure 2A shows that only one copy of the expression cassette was integrated in the genome. TAIL-PCR reactions led to the identification of the gene responsible for the observed phenotype. This gene shared 45% and 44% identity with the Nmr repressors *nmr-1* of *N. crassa*, and *nmrA* of *A. nidulans*, respectively, and was further named *OmNmr1*. The hygromycin gene, used for codon cassette mutagenesis, interrupted the gene at 435 bp downstream of the start codon (Fig. 2B).

Previous studies reported an increased sensitivity to chlorate, a toxic analogue of nitrate, and thiourea, a toxic analogue of urea, in the presence of ammonium in *A. nidulans* and *N. crassa nmr* mutants (Andrianopoulos *et al.*, 1998; Jarai and Marzluf, 1990). In the wild type, no significant growth difference was observed between the control and toxic analogues-amended media (Fig. 2C). By contrast, the *OmNmr1* mutant showed a high sensitivity to both chlorate and thiourea in respect to the control (Fig. 2C).

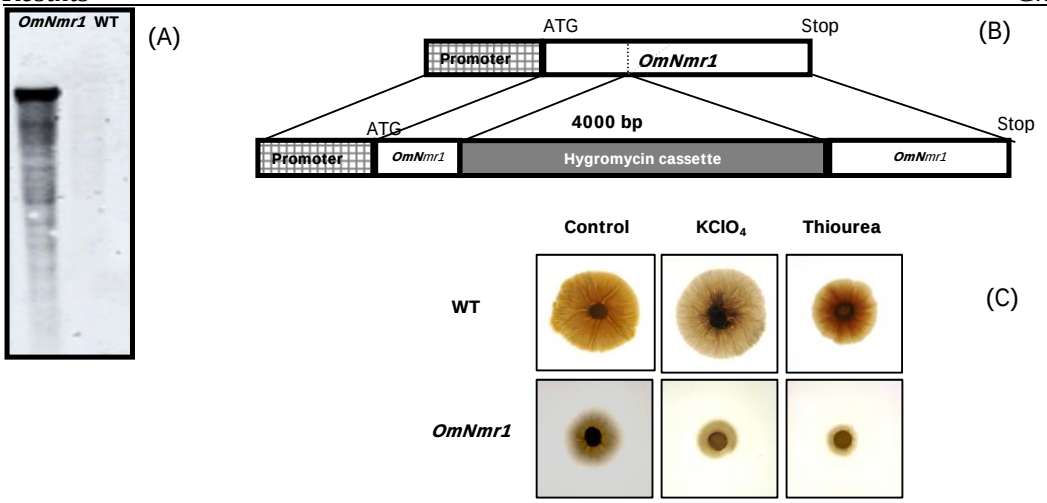

Figure 2. (A) Southern blot analysis. The genomic DNA of the *OmNmr1* and the WT were digested with *BglII* restriction enzyme and were size-fractionated on a 1% (w/v) agarose gel. The blot was probed with a fragment of the hygromycin-resistance gene, and the hybridization was performed with a chemiluminescent detection system. (B) hygromycin cassette insertion within the *OmNmr1* gene. (C) Growth assays for *O. maius* WT and for the *OmNmr1* mutant on 125 mM potassium perchlorate ($KClO_4$) or 4 mM thiourea, in the presence of ammonium tartrate as a nitrogen source instead of $NaNO_3$.

3.3. *OmNmr1* mutation induces a modification of the free amino acids pools

Free amino acids were quantified for both the WT and the *OmNmr1* mutant using GC-MS. Before nitrogen starvation, glutamine, glutamate, alanine and asparagine were the most abundant amino acids measured in the mycelium, both in the WT and in the mutant. The WT and the mutant had comparable values for these amino acids, with the exception of alanine levels which were higher in the WT compared to the mutant (Fig. 3). Nitrogen starvation did not have any effect on the glutamate content neither in the WT nor in the mutant, whereas, it led to a decrease in the glutamine content which was more pronounced in the mutant in respect to the WT. The alanine slightly decreased in the WT, whereas it increased in the mutant. The asparagine showed an opposite trend as it increased in the WT and decreased in the mutant (Fig. 3). After the nitrogen re-feeding period, the amount of glutamine and asparagine in the *OmNmr1* mutant remained lower than in the WT. By contrast, the concentrations of glutamate and alanine increased in the *OmNmr1* mutant and were higher than those of the WT (Fig. 3).

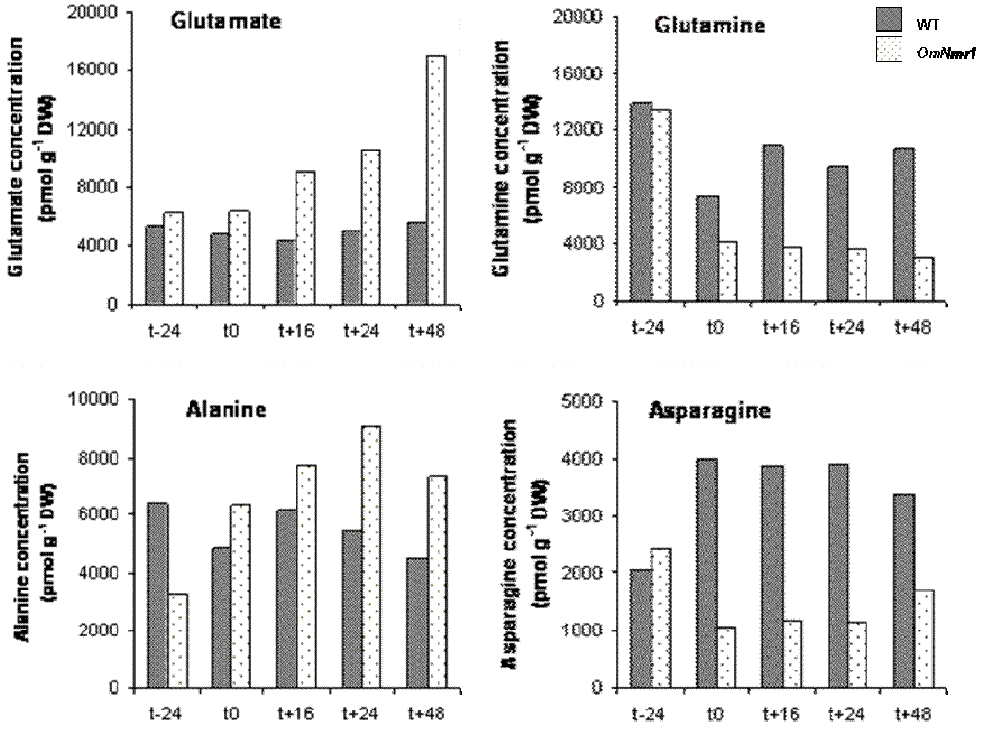

Figure 3. Amino acid contents (pmol g^{-1} DW) in *O. maius* Zn WT and *OmNmr1* mutant measured through GC-MS. Both the WT and the *OmNmr1* mutant were grown for 30 days in liquid Czapek-glucose medium with NO_3 as a nitrogen source and starved from nitrogen for 1 day. Nitrate was then re-supplied to the medium at a 35 mM concentration and mycelia were harvested after 16h, 24h and 48h. Glutamate, alanine, glutamine, and asparagine were quantified using GC-MS before N starvation (t-24), N starvation (t0), and N re-feeding after16h (t+16), 24h (t+24), and 48h (t+48).

3.4. *OmNmr1* mutation induces a striking reduction of the GS activity

The GS and the GDH activities were measured for the WT and the mutant grown in the presence of different nitrogen sources. In the WT, the GS specific activity was significantly higher when it was grown on the medium containing $NaNO_3$ as compared to the other nitrogen sources (Fig. 4A). Most interestingly, in all growth conditions tested, the GS activity of the mutant strain was always lower than that of the WT strain, at almost undetectable levels (Fig. 4A). By comparing with the WT, a reduction of the mutant GS activity by 95%, 72%, 85%, 37% and 73% was measured on media supplemented with $NaNO_3$, $(NH_4)_2SO_4$, NH_4 tartrate, glutamine and glutamate, respectively (Fig. 4A). On the contrary, on all the nitrogen sources tested, the mutant GDH specific activity was always higher when compared to the WT activity (Fig. 4B).

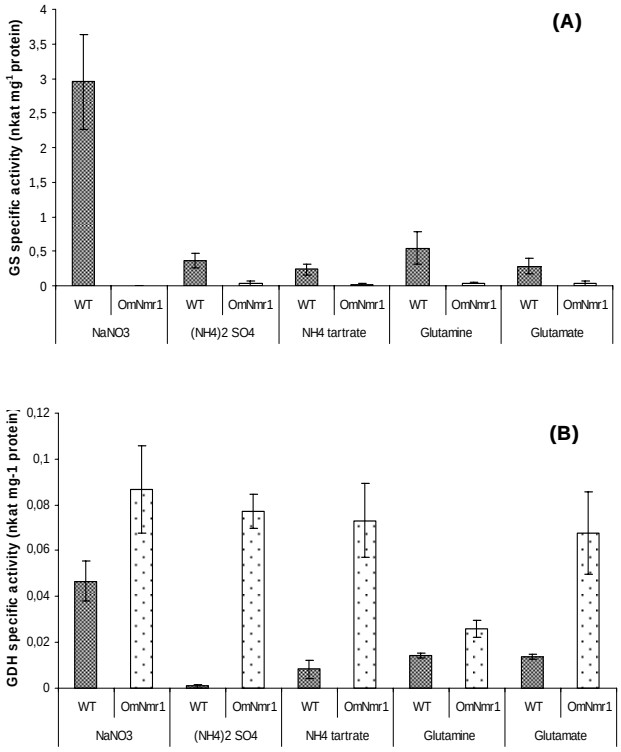

Figure 4. (A) Glutamine synthetase (GS) (nkat mg^{-1} protein) and (B) NADP-glutamate dehydrogenase (GDH) (nkat mg^{-1} protein) specific activities of the WT and the *OmNmr1* mutant grown on solid Czapek-glucose medium supplemented with 0.49 g/l of the following N sources: $NaNO_3$, $(NH_4)_2SO_4$, NH_4 tartrate, glutamine, or glutamate. Soluble protein content was measured by a modification of the Bradford method (1976). GS activity was determined by a modification of the transferase assay of Shapiro and Stadtman (1970). NADP-GDH activity was performed by following NADPH oxidation at 340 nm.

3.5. Exogenous glutamine compensates growth defect of the mutant

As the level of glutamine measured for the *OmNmr1* mutant was low, we checked whether the presence of glutamine in the medium could compensate the defect of endogenous glutamine biosynthesis. Both the WT and the mutant were inoculated on different nitrogen sources (Fig. 5A) and colony phenotypes and biomasses were evaluated. For the WT, glutamate was the best nitrogen source for mycelium growth, followed by $(NH_4)_2SO_4$, glutamine, NH_4 tartrate and $NaNO_3$ (Table 1). For the *OmNmr1* mutant, the mycelium biomass was significantly reduced on all the nitrogen sources if compared to the WT, but the reduction rate varied with the nitrogen source (Table 1). A significant increase of the *OmNmr1* mutant mycelium biomass was measured on glutamine containing medium. The ratio *OmNmr1* mutant/WT showed that the mycelium growth of the mutant was quite similar to that of the WT only when the glutamine was used as nitrogen source (Fig. 5B). The mycelium biomasses on nitrate and glutamate were similar, and the most pronounced growth reduction was observed on medium containing ammonium as a nitrogen source.

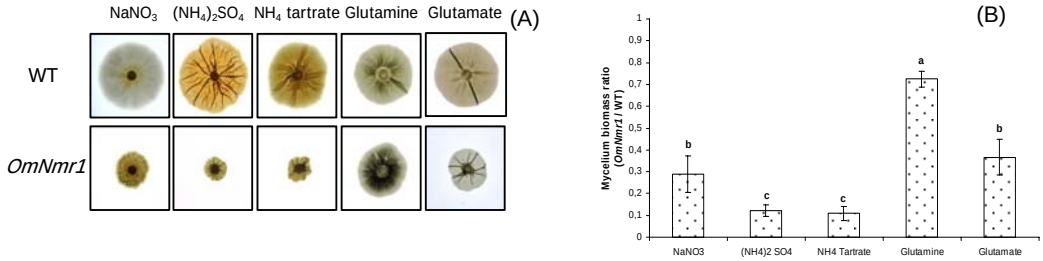

Figure 5. (A) Mycelium growth of the WT and the *OmNmr1* mutant on solid Czapek-glucose medium containing 0.49 g/l of the following N sources: $NaNO_3$, $(NH_4)_2SO_4$, NH_4 tartrate, glutamine, or glutamate. (B) Mycelium biomass ratio between the *OmNmr1* mutant and WT. Prior to inoculation, sterile cellophane membranes were placed aseptically on the agar surface. Fungal colonies (3 replicates/treatment) were removed after 30 days by peeling the biomass from the cellophane membranes, and biomasses were recorded.

Table 1. Dry mycelium biomass (mg) of the WT and the *OmNmr1* mutant grown on different nitrogen sources.

N. sources	$NaNO_3$	$(NH_4)_2SO_4$	NH_4 tartrate	Glutamine	Glutamate
WT	87.77 ± 5.20	138.20 ± 2.06	108.27 ± 5.43	121.70 ± 0.73	154.67 ± 1.59
OmNmr1	25.40 ± 5.92	16.93 ± 3.08	11.77 ± 2.88	88.23 ± 3.67	56.60 ± 10.24

3.6. Mycorrhiza formation

We checked whether the mutation of the *OmNmr1* gene could alter the ability of the fungus to establish a mycorrhizal symbiosis with roots of its host plant. Microscopic observations showed no difference concerning the percentage of mycorrhization between the WT and the *OmNmr1* mutant, suggesting that the mutation did not alter the ability to establish the mycorrhizal association (data not shown).

4. Discussion

Insertional mutagenesis is a powerful tool to study specific gene function as it allowed the generation of loss-of-function mutations. In the present study, we report the characterization of the *OmNmr1* mutant. The mutation is the result of the insertion of a hygromycin cassette within the *OmNmr1* gene. Similarly to *nmr-1*Δ and *nmrA*Δ (Jarai and Marzluf, 1990; Andrianoloulos *et al.*, 1998), the mutation of *OmNmr1* resulted in a sensitivity to chlorate and thiourea suggesting the inactivation of this gene and its involvement in nitrogen regulation.

In *A. nidulans* the NmrA expression is repressed under nitrogen starvation and, as the AreA level increased, an upregulation of the AreA target genes such as the glutamine synthetase and the nitrate reductase encoding genes is observed. On the contrary, under nitrogen-sufficient conditions, the nmrA level increases, preventing AreA function (Andrianopoulos *et al*., 1998). In *A. nidulans*, AreA accumulates in the nucleus during nitrogen starvation and is exported from the nucleus and complexes with NmrA when nitrogen-riched sources are added to starving mycelia (Todd *et al*., 2005). Deletion of the *nmrA* gene induces a derepression of the AreA target genes, showing a similar phenotype than strains grown under nitrogen starvation conditions.

As the *nmr* gene is reported to be involved in the NMR mechanism, we evaluated the role of *OmNmr1* gene in the nitrogen metabolism. Considering data obtained for amino acid pools as well as GS and GDH activities, the *OmNmr1* gene seems to play a key role in the control of the nitrogen metabolism in *O. maius*. The mutant, when compared to the WT, was indeed characterized by a higher production of glutamate and alanine, while reduced amounts of glutamine and asparagine were measured. These observations are similar to those reported for some ectomycorrhizal fungi in the presence of a GS inhibitor (Chalot *et al.*, 1991; Martin *et al*., 1994; Turnbull *et al*., 1996; Morel *et al*., 2006). We therefore hypothesized that the *OmNmr1* gene may somehow interact with the GS activity, an enzyme that is absolutely required for the biosynthesis of glutamine (Teichert *et al*., 2004). Our hypothesis was confirmed by the evaluation of GS and GDH activity in *O. maius*. In fact, on all nitrogen sources tested, GS specific activity in the mutant was severely reduced or even undetectable. The reduced level of the GS specific activity in the mutant could be the direct consequence of the loss of the *OmNmr1* gene function. A complementation of the mutant with the *OmNmr1* gene could confirm this point.

The high amount of glutamate in the mutant as compared to the WT suggests that nitrogen can cycle through another pathway, namely the NADP-GDH. Morel and colleagues (2006) distinguished two groups of fungi. Group I (GDH^+ fungi) includes fungi with both NADP-

GDH and GS activities and Group II (GDH^- fungi) with no detectable NADP-GDH activity and with GS as the unique pathway for ammonium assimilation. *O. maius* possesses the two enzyme activities and therefore belongs to the first group (GDH^+) of fungi, which also includes *Hebeloma cylindrosporum*, *Cenococcum geophilum*, *Tuber borchii* and *Laccaria bicolor*, with a GS activity significantly higher when compared to the NADP-GDH activity.

To our knowledge, the effect of the *nmr* gene on the activity of the nitrogen assimilatory enzymes has been reported only for the yeast *Hansenula polymorpha* (Serrani *et al.*, 2001). The GS activity in the *nmr1-1* mutant of *H. polymorpha* was comparable or even higher than the GS WT activity. Serrani and collaborators (2001) hypothesized that *nmr1-1* was a very leaky mutation in the GS gene. Similar mutants have been described in *A. nidulans* (Arst *et al*., 1982) and in *N. crassa* (Dunn-Coleman *et al*., 1979). These mutants showed a clear-cut of the nitrogen metabolite derepression, while manifesting no noticeable glutamine auxotrophy. Serrani *et al*. (2001) assumed that since *H. polymorpha* seemed to exclude a GS defect, glutamate (or a derivative of this compound) might be involved in signaling nitrogen metabolite repression. In our case the opposite situation was observed, with a highly reduced GS activity in the *OmNmr1* mutant, coupled to a much higher GDH activity. We can therefore assume that for the *OmNmr1* mutant, glutamine and not glutamate is involved in signaling nitrogen metabolite repression. Glutamine appears to be the critical metabolite that acts as a nitrogen catabolite repressor (Marzluf, 1997).

Christensen *et al.* (1998) reported the determination of the levels of the GDH and GS enzymes in *areA* mutants of both *A. oryzae* and *A. nidulans*. In both cases the NADP-GDH levels were reduced, whereas the GS levels were not affected. These results suggest that the AreA protein may play an important role in the regulation of nitrogen assimilation in addition to its previously established regulatory role in nitrogen catabolism (Christensen *et al.*, 1998). They also measured levels of GDH and GS activities in WT and *areA* deletion strains of both *A. oryzae* and *A. nidulans*: the levels of GDH activity were low on glutamate and high on glutamine, whereas the levels of GS activity were low on glutamine and high on glutamate. The levels of GDH activity were substantially reduced in the *areA* mutant strains of both species compared to those in the respective WT strains on ammonium, whereas the levels of GS activity were not affected. Christensen *et al.* (1998) studied an *areA* mutant so the phenotypic effect cannot be compare with those obtained for a *nmr* mutant, as AreA and NmrA proteins act in an antagonist way (Schönig *et al.*, 2008). Moreover, the mRNA levels of areA and nmrA are inversely regulated, suggesting that their relative levels are critical in determining AreA activation (Wong *et al*., 2007).

In *A. nidulans* and *N. crassa*, the deletion of *nmrA* and *nmr-1*, respectively, led to a significant derepression of AreA target genes (Andrianopoulos *et al.*, 1998; Jarai and Marzluf, 1990). In particular, deletion of the *nmr* gene generally induces an upregulation of the *glnA* gene, which encodes the GS enzyme, when the mutant is incubated with glutamine (Schönig *et al.*, 2008; Wagner *et al.*, 2010).

On the contrary, in the *OmNmr1* mutant the mutation induced a reduction of the GS activity. The results reported for *A. nidulans* and for *N. crassa* refer to the transcriptional level, while our results describe the activity of the enzyme, so an evaluation of the GS gene expression is required to fully compare our results with those reported in literature. Furthermore, a lack of a direct correlation between the GS activity and the GS mRNA levels should be taken into consideration (Breuninger *et al.*, 2004).

We could also explain our results assuming that the reduction of the GS activity is the result of a negative feedback mechanism in which the production of a high amount of glutamine in the first steps of growth could inhibit the GS activity in the late growth phase. A time course of the GS activity in the different growth fungal phases could help in clarifying this point.

Ammonium and glutamine are considered as the preferred nitrogen source for fungi (Mikes *et al*., 1994). As the *OmNmr1* mutant cannot synthesize glutamine, if not at a very low level, we checked whether exogenously supplied glutamine could compensate the defect of glutamine biosynthesis and improve mutant growth. The WT and the *OmNmr1* mutant were inoculated on media containing different nitrogen sources. An increased of the mutant mycelium biomass was measured on glutamine containing medium as compared to other nitrogen sources. Moreover, unlike ammonium, on which the *OmNmr1* mutant displayed a reduced growth, exogenous supplied glutamine enabled the mutant to grow at the same level as that of the WT. This phenotype was previously observed in the *areA*Δ mutant of *A. oryzae* (Christensen *et al.*, 1998) and also in the mutant of *Microsporum canis* lacking the *areA*-like nitrogen regulatory gene (*dnr1*) (Yamada *et al.*, 2006). Similarly, Cornwell and MacDonald (1984) observed that *glnA* mutants of *A. nidulans* require glutamine supplementation for growth. However, even in the presence of glutamine, the *OmNmr1* mutant exhibited a slightly reduced biomass as compared to the WT. One explanation could rely on the fact that endogenously synthesized and exogenously supplied glutamine pools are not equivalent (Wiame *et al.*, 1985). The reduced growth of the mutant on the other nitrogen sources tested could be explained by the accumulation of glutamate due to the reduced GS activity. In fact, the loss of the ability to recycle glutamate into glutamine may result in increased glutamate levels, leading to toxic effects within cells (Margelis *et al.*, 2001).

Nitrogen uptake by plants is greatly improved by the mutualistic associations with mycorrhizal fungi. Previous studies reported the importance of arginine in arbuscular mycorrhizal fungi (Govindarajulu *et al.*, 2005) and glutamate, glutamine, aspartic acid, asparagine in ectomycorrhizal fungi (Finlay *et al.*, 1988) as the amino acids involved in nitrogen translocation to plant roots. As the *OmNmr1* gene had a negative effect on glutamine and asparagine biosynthesis, we could expect an effect on mycorrhization process (i.e. less vigorous plants when mycorrhized with the *OmNmr1* mutant), but no difference was observed, neither on viability of the WT and the mutant mycorrhized plants, nor on mycorrhizal ability of the *OmNmr1* mutant. We could therefore hypothesize that the overproduction of glutamate measured for the *OmNmr1* mutant can largely compensate, during the mycorrhization process, for the low glutamine and asparagine biosynthesis.

To our knowledge, this is the first work illustrating the generation of an *nmr* mutant in a mycorrhizal fungus. This mutant was isolated from a screening for oxidative stress tolerance (by using cadmium, zinc and menadione) of a random-mutant collection of *O. maius* strain Zn.

Toxic heavy metals negatively affect plants and microorganisms (Prasad and Hagemeyer 1999). Metal toxicity involves oxidative and/or genotoxic mechanisms (Briat and Lebrun, 1999). In plants and microorganisms, polypeptides and proteins such as ferritins and metallothioneins, and glutathione-derived peptides named phytochelatins, participate in excess metal storage and detoxification. Low molecular weight organic molecules, mainly organic acids and amino acids and their derivatives, also play an important role in metal homeostasis. When these systems are overloaded, oxidative stress defense mechanisms are activated. Living cells possess several mechanisms for maintenance of the redox balance. This is known as the redox homeostasis, which is maintained by antioxidant defense systems and their associated regulation pathways (Briat and Lebrun, 1999). As a defense system against oxidative stress, cells possess a range of non-enzymatic and enzymatic defense systems including molecular scavengers (i.e., glutathione, thioredoxin, and glutaredoxin) and detoxifying enzymes (catalase, superoxide dismutase, and peroxidase) (Hradilová *et al.*, 2010).

Considerable effects of metals and the related oxidative stress on the nitrogen metabolism have been reported (Boussama *et al.*, 1999). Nitrogen metabolism is the basis for an optimal biosynthesis of amino acids and proteins, which are involved in nearly all processes in living organisms (Forde and Clarkson 1999). Some amino acids have also been shown to play a significant role in metal chelation and tolerance in plants (Hall, 2002; Sharma and Dietz, 2006).

The *OmNmr1* mutant obtained in the present study was shown to be highly sensitive to oxidative stress inducing substances. This mutant was characterized by a very low glutamine and asparagine content and by a higher amount of glutamate and alanine. The low glutamine and asparagine content is mirrored by a very low, almost undetectable, GS activity.

A number of studies reported the involvement of glutamine and GS enzyme activity in the tolerance mechanisms to metal caused oxidative stress. These studies could therefore help in understating why the *OmNmr1* mutant was identified among the most sensitive ones through a random-mutant screening for oxidative stress.

The *OmNmr1* mutant was shown to have very low GS activity and glutamine content. Glutamine is the most abundant free amino acid in the cell and known to play a regulatory role in several cell-specific processes (Lee *et al*., 2007). Glutamine synthetase is a key metabolic enzyme that converts glutamine into a nitrogen source for pathway leading to biosynthesis of amino acids, nucleic acids, and complex polysaccharides. GS plays a role in the flow of nitrogen into nitrogenous organic compounds by catalyzing the assimilation of ammonia into glutamine, which is then converted to glutamate via the action of glutamate synthase (Li *et al*., 1993; Rana *et al*., 2008). Glutamine plays a major role in cell proliferation and provides protection from apoptotic stimuli (Lee *et al*. 2007), regulates the cellular redox balance, including oxidative metabolism, and cell proliferation in mammalian cells (Matés *et al*. 2002). Glutamine is also a substrate for protein synthesis, an inter organ nitrogen transporter and a precursor for glutathione production. Many of these functions are connected to the formation of glutamate from glutamine (Matés *et al*., 2002). Therefore, the glutamine/glutamate balance may play a central role in heavy metal tolerance and accumulation.

A possible role of glutathione is to reduce the concentration of free metal ions in the cell, thus preventing an increased production of reactive oxygen species (ROS) under heavy metal stress (Xu *et al*., 2009). Glutamine has been reported to mediate the biosynthesis of the cellular antioxidant glutathione, so it has an important role in the regulation of the cellular redox balance (Matés *et al*., 2002).

The low glutamine production and the low GS activity in the *OmNmr1* mutant could affect the biosynthesis of glutathione, and this observation could therefore partly explain the high sensitivity of the mutant to Zn, Cd, and menadione. This mutant represents therefore a very useful instrument to study both nitrogen metabolism and oxidative stress tolerance in the ericoid fungus *O. maius.*

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Chapter 6

GENERAL CONCLUSIONS

The increasing contamination of heavy metals due to anthropogenic activities, such as industry, agriculture, mining, transportation, construction and habitations, are dangerously affecting the biological ecosystems. The most critical feature of heavy metals is that they are not biodegradable (Rauret *et al.*, 1999), and they represent a serious threat for the biological systems and for human health. Numerous remediation technologies have been developed to reduce the risk of these pollutants, but the remediation techniques employing living organisms (bioremediation) are generally considered the cheapest and the most environmental friendly.

Mycorrhizal associations, including arbuscular, ecto- and ericoid mycorrhizas, provide an attractive system to advance plant-based phytoremediation of heavy metals (Van der Lelie *et al*., 2001; Rosen *et al*., 2005, Gohre and Paszkowski, 2006). In fact, mycorrhizal fungi may actively participate to the phytoremediation process, and may be useful in extending the application of phytoremediation to contaminated sites (Kuffner *et al.*, 2008). Mycorrhizal fungi may both increase and reduce plant metal accumulation, and may enhance phytoextraction directly or indirectly by increasing plant biomass (Tullio *et al*., 2003). The potential impact of mycorrhizal fungi on bioremediation is dependent on the physical and chemical nature of the contaminated soils (Martino and Perotto, 2010), on the mycorrhizal type, and particularly on the degree of metal tolerance of the fungal strains (Gadd, 2010).

The mechanisms by which mycorrhizal fungi survive and tolerate toxic concentrations of heavy metals, as well as the mechanisms that protect the host plants from metal toxicity are, however, poorly understood.

The fungal isolate investigated in this thesis is the ericoid fungus *Oidiodendron maius* (Barron, 1962) strain Zn, deposited in the MUT collection at the Department of Plant Biology, University of Turin (MUT 1381). This strain was isolated from the Niepolomice Forest (25 km N.E. of Krakow, Poland) from the roots of *Vaccinium myrtillus* growing in experimental plots treated with 5000 t km^{-2} of dusts containing high concentrations of zinc, cadmium and aluminium derived from industrial electro-filters (Martino *et al*., 2000). Although the mechanisms are unclear, ericoid fungi have been shown to protect their host plants from the adverse effects of heavy metals (Bradley and Read, 1981; Denny and Ridge, 1995; Sharples *et al*., 2000) and the colonization of the Niepolomice Forest plots by ericoid mycorrhizal plants suggests that *O. maius* can confer to the host plant the ability to survive in metal polluted environments.

O. maius Zn has been demonstrated to tolerate concentrations of zinc that cause a complete growth inhibition of isolates from unpolluted soils (Martino *et al*., 2000). The comprehension of metal homeostasis/tolerance mechanisms for this fungus is critical, because it may help to

understand how fungal tolerant strains cope with metal stress, and, eventually, how they are involved in the protection of the host plants from heavy metal toxicity.

The main aim of the thesis was the identification of genes involved in zinc tolerance of *O. maius* strain Zn. Gene-knock out, yeast mutant functional complementation and random-mutant screening successfully led to the identification/characterization of six genes that are potentially involved in oxidative/metal stress response (Fig. 1).

In fungi, the mechanisms potentially involved in metal tolerance can act both in the intra- and in the extra-cellular environment. Extracellular mechanisms consist mainly in the reduction of metal uptake through organic acid-chelation, binding of metals to extracellular proteins, and adsorption to the fungal cell wall. Despite the extracellular mechanisms to avoid metal influx, metal ions can enter into cells, especially when the mycelium is exposed to high metal concentrations. One strategy to cope with intracellular metal toxicity consists in the chelation of metal ions in the cytosol. Metallothioniens (MTs) and phytochelatins (PCs) represent the main chelators of metal ions in the cytosol and contribute to the buffering of toxic metal concentration within cells. MTs have been demonstrated to be a potential determinant in the response to metal toxicity in mycorrhizal fungi, such as *Gigaspora margarita* (Lanfranco *et al.*, 2002), *Paxillus involutus* (Bellion *et al*., 2007) and *Hebeloma cylindrosporum* (Ramesh *et al*., 2009), while, no phytochelatins were found in *P. involutus* (Courbot *et al.*, 2004), *O. maius* (Vallino *et al*., 2005), and *Laccaria laccata* (Galli *et al*., 1993). Phytochelatins have been recently found in *Tuber melanosporum* (Bolchi *et al*., 2011).

Mechanisms of metal detoxification are also based on the prevention of metal accumulation in the cytosol. Specific transporters are responsible for the uptake and export of metal ions, and for their sequestration into organelles. However, only few transporters have been isolated and characterized in mycorrhizal fungi. For example, the two Cu-transporters *GintABC1* (González-Guerrero *et al.*, 2007) and P-type Cu-ATPase (Benabdellah *et al*., 2007) have been identified on the tonoplast of *Glomus intraradices*. In the same fungus, the first zinc transporter described in arbuscular mycorrhizal fungi (*GintZnT1*) has been isolated and has been shown to contribute to Zn sequestration and tolerance (González-Guerrero *et al.*, 2005). Blaudez and Chalot (2011) have recently isolated the *HcZnt1* gene coding for a CDF Zn transporter in *H. cylindrosporum*, and reported its role in Zn homeostasis and tolerance. Here, we report the identification and the partial characterization of the first zinc transporters in an ericoid mycorrhizal fungus. Two full-length cDNAs from *O. maius* Zn were isolated and named *OmCDF* and *OmFET*. The heterologous expression of *OmCDF*, coding for a CDF transporter, in different *S. cerevisiae* mutants showed that it was able to complement the Zn-, Fe- and Co-sensitive phenotypes, but not the Cd- and the Mn-sensitive phenotypes. The

OmCDF protein is targeted to the endoplasmic reticulum (ER) membranes, as shown for the *S. pombe* Zhf1 (Clemens *et al*., 2002) and the *H. cylindrosporum* Znt1 (Blaudez and Chalot, 2011). We suggest for OmCDF the same mechanism reported for the *S. pombe* Zhf1 and for the *H. cylindrosporum* Znt1, in which both transporters play an important role in metal tolerance and are localized on the ER membranes, having the ER a major role in metal storage (Clemens *et al*., 2002; Blaudez and Chalot, 2011).

OmFET restored the growth of uptake-defective strains for Zn, Fe and Mn, and the OmFET-GFP fusion protein was located on the plasma membrane. *OmFET* may act as an important path for Fe entry into Fe-deficient cells. Phenotype complementation of the Zn-sensitive yeast mutant with *OmFET* suggests that the zinc tolerance mechanism could derive from its ability to take up Fe, therefore counteracting Zn toxicity. This hypothesis is in accordance to literature data reporting a fine tuned coordination of zinc and iron metabolism and a decreased zinc toxicity under high iron concentrations (Yasmin *et al*., 2009; Pagani *et al*., 2007). We can suggest that these two fungal transporters involved in metal homeostasis and tolerance could also play a role in reducing the plant uptake of heavy metals during the symbiosis.

The screening of a collection of *O. maius* random-mutants on Zn, Cd and menadione aimed to identify hypersensitive mutants led to the identification of a transporter, named *OmMFS*, belonging to the major facilitator superfamily (MFS). MFS proteins facilitate the transport across the plasma membrane, or internal membranes, of a variety of substrates including ions, sugar phosphates, drugs, amino acids, and peptides (Pao *et al.*, 1998; Walmsley *et al.*, 1998). This gene could play an important role in the metal tolerance mechanisms, as previously reported in a number of studies concerning filamentous fungi (Hayashi *et al.*, 2002; Morschhäuser, 2010), yeast (Panwar *et al.*, 2008), and plants (Haydon and Cobbett, 2007; Cabrito *et al.*, 2009).

Once entered into the cell, metals can cause oxidative damages both directly, by increasing the level of reactive oxygen species (ROS), and indirectly, by leading to a severe depletion of free-radical scavengers such as thiol-containing compounds. Therefore, antioxidant defense mechanisms are in place to maintain ROS at a basal level (Fridovich, 1998). These mechanisms include enzymatic antioxidants such as superoxide dismutases, catalases, peroxidases and glutaredoxins (Yu, 1994; Neill *et al*., 2002), as well as non-enzymatic antioxidants such as glutathione (GSH) and vitamins (Evans and Halliwell, 2001).

As described in many fungi, the superoxide dismutase has a twofold importance. It has been proposed to play a role in plant-fungal interactions (Hammond-Kosack and Jones, 1996) and it is a key enzyme in the detoxification of the cell from ROS (Gralla and Valentine, 1991; Guelfi *et al*., 2003; Todorova *et al*., 2007; Jacob *et al*., 2001; Tarhan *et al*., 2007). However,

there are only few reports on this gene in mycorrhizal fungi. In the ectomycorrhizal fungus *P. involutus*, Jacob and colleagues (2001) isolated a full-length cDNA encoding a SOD enzyme, and observed that this gene rescues the transformed *E. coli*-SOD null mutant from oxidative stress generated by paraquat and cadmium. Lanfranco *et al*. (2005) found that the Cu,Zn superoxide dismutase of the arbuscular mycorrhizal fungus *Gigaspora margarita* is up-regulated during symbiosis with legume hosts. The Cu,Zn superoxide dismutase (Cu,Zn-SOD) was isolated and characterized for *O. maius* Zn starting from a cDNA library constructed by using mRNA from Zn-treated *O. maius* mycelia (Vallino *et al*., 2005). Functional complementation assays showed that *OmSOD1* confers increased tolerance to zinc and copper stress to a Cu,Zn SOD-defective yeast mutant (Vallino *et al*., 2009). The OmSod1 protein was found both in the cell extract and in the growth medium of viable fungal cultures. Here, we report the first Cu,Zn SOD-targeted disruption in a mycorrhizal fungus via homologous recombination. The disruption of *OmSOD1* resulted in the presence of oxidative stress markers, even in the absence of external stress. In addition, phenotype characterization of SOD1-null mutants revealed that the inactivation of *OmSOD1* gene induced an increased sensitivity to Zn, Cd, and especially to menadione with respect to the *wild-type*. Moreover, the deletion of *OmSOD1* gene induced a reduction in conidiation and in the percentage of mycorrhization of *V. myrtillus* roots. Therefore, the SOD1 protein appeared to have a double role being involved not only in fungal protection against oxidative stress, but also in the fungus-plant interaction. Its disruption could have caused an imbalance in the redox homeostasis during host colonization and an alteration in the delicate dialogue between the fungus and its host plant. In agreement with this result, an induction of ROS-scavenging enzymes have been reported also in AM associations (Salzer *et al*., 1999; Tamasloukht *et al*., 2003; Fester and Hause 2005; Lanfranco *et al*., 2005; Seddas *et al*., 2009).

Glutathione (GSH) is one of the most abundant low molecular weight thiols, and it is thought to play a vital role in buffering the cell against ROS (Stephen and Jamieson, 1996). A possible role of glutathione is to reduce the concentration of free metal ions in the cell, thus preventing an increased production of reactive oxygen species under heavy metal stress (Xu *et al*., 2009). Glutamine has been reported to mediate the biosynthesis of the cellular antioxidant glutathione, so it has an important role in the regulation of the cellular redox balance (Matés *et al*. 2002). Here, we report the characterization of an *O. maius*-mutant that carries a mutation in the *nmr* (nitrogen metabolite repression) gene. This mutant was isolated through a screening of *O. maius* random-mutants for oxidative stress responses. The free amino acid pools, the glutamine synthethase (GS) and glutamate dehydrogenase (GDH) enzyme activities were measured both in the wild type and in the mutant. In the mutant, an increase of

glutamate and alanine contents and a decrease of glutamine and asparagine were recorded. In parallel assays, GS activity resulted significantly reduced in the mutant and compensated by GDH activity. These enzymatic activities could explain the reduced concentration of glutamine and asparagine. Moreover, the addition of glutamine in the medium determined an increase in the mycelium mutant biomass that was not observed on other nitrogen sources. The low glutamine production and the low GS activity in the mutant could affect the biosynthesis of glutathione, and this observation could therefore partly explain the high sensitivity of the mutant to Zn, Cd, and menadione. This mutant represents therefore a very useful instrument to study both nitrogen metabolism and oxidative stress tolerance in the ericoid fungus *O. maius.* Considerable effects of metals, and the related oxidative stress, on the nitrogen metabolism have in fact been reported (Boussama *et al*. 1999). Nitrogen metabolism is the basis for optimal biosynthesis of amino acids and proteins (Forde and Clarkson 1999), and some amino acids have been shown to play a significant role in metal chelation and tolerance (Hall, 2002; Sharma and Dietz, 2006).

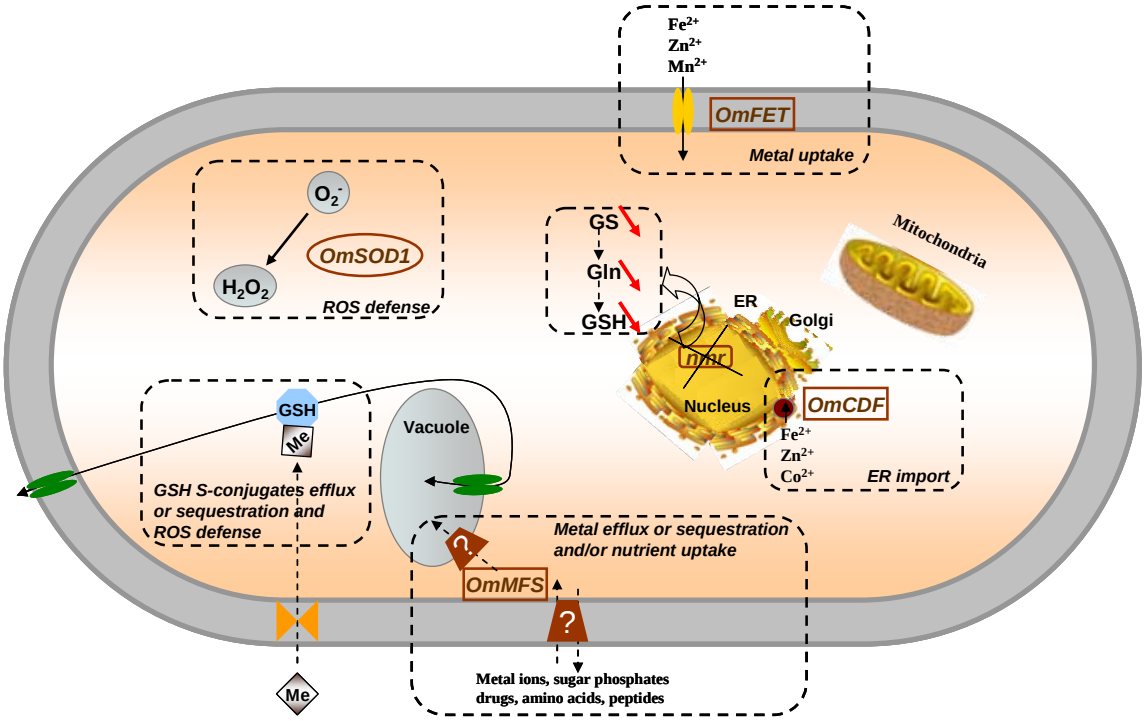

Figure 1. Schematic representation of the different mechanisms involved in heavy metal and oxidative stress tolerance in the ericoid mycorrhizal strain *Oidiodendron maius* Zn reported in this thesis. ER: endoplasmic reticulum; Gln: glutamine; GS: glutamine synthetase; GSH: glutathione; Me: metal; nmr: nitrogen metabolite repression; ROS: reactive oxygen species.

In conclusion, *Oidiodendron maius* has been developed as a model system to investigate metal tolerance in mycorrhizal fungi. The use of non targeted functional assays, such as complementation of yeast mutants with *O. maius* cDNA libraries and screening of *O. maius* random mutants, has been instrumental for the identification of genes with diverse functions (e.g. enzymes, transporters, transcriptional regulators) involved in zinc tolerance in this metal-tolerant ericoid mycorrhizal fungus. The results described in this thesis shed some light on the extreme complexity underlying the tolerance mechanisms in *O. maius*, showing a relationship between nitrogen and zinc metabolism, and add new useful elements to understand the stress response puzzle. High throughput transcriptomic data and sequencing of the entire genome are becoming available for *O. maius* strain Zn, thus making this model system an even more powerful tool to genetically dissect metal tolerance in mycorrhizal fungi.

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Molecular approaches to study oxidative stress tolerance mechanisms in the ericoid mycorrhizal fungus *Oidiodendron maius*

Abstract
Due to increasing anthropogenic activities, large areas are highly contaminated by heavy metals which are affecting biological systems. *Oidiodendron maius* strain Zn could be an interesting organism in a bioremediation program being both an ericoid mycorrhizal fungus and a heavy metal-tolerant strain. To understand the mechanisms underlying the oxidative stress tolerance of this strain, three different approaches were used.
The first approach allowed us to obtain superoxide dismutase 1 (SOD1) null mutants. The most important technical advance in this work was the first successful disruption of a gene by homologous recombination in a mycorrhizal fungus. We demonstrate that the lack of *OmSOD* may cause an imbalance in the redox homeostasis and an alteration in the delicate dialogue between the fungus and its host plant.
The second approach was based on a yeast functional complementation screening using an *O. maius* cDNA library. In this work we report the first transporters of an ericoid mycorrhizal fungus capable of conferring Zn tolerance to yeast transformants. Two full-length cDNAs were isolated and named *OmCDF* and *OmFET*. The heterologous expression of these two genes in various yeast mutants conferred resistance to zinc. Additionally, *OmCDF* expression also conferred Co tolerance. We provide evidence that OmCDF functions as a Zn transporter responsible for relocating cytoplasmic Zn into the endoplasmic reticulum, whereas expression of *OmFET* could counteract Zn toxicity by increasing Fe content of cells.
The third approach consisted in the screening of a collection of *O. maius* random-mutants on Zn, Cd and menadione. We report the characterization of an *O. maius*-mutant that carries a mutation in the *nmr* gene. In this mutant, a decrease of glutamine and asparagine pools, and a reduction of the activity of glutamine synthase were recorded. Possible links between the oxidative stress tolerance and the nitrogen metabolism are discussed.

Keywords: ericoid mycorrhizal fungus, tolerance mechanisms, heavy metals, oxidative stress.

Identification par approches moléculaires de gènes impliqués dans la tolérance au stress oxydatif chez le champignon mycorhizien *Oidiodendron maius*

Résumé
En raison des activités anthropiques croissantes, de larges sites sont contaminés par les métaux lourds qui affectent les systèmes biologiques. La souche *Oidiodendron maius* Zn pourrait être un organisme intéressant dans un programme de bioremédiation étant à la fois un champignon mycorhizien éricoïde et une souche tolérante aux métaux lourds. Pour comprendre les mécanismes de la tolérance de cette souche, trois approches différentes ont été menées.
La première approche a abouti à la génération de mutants du gène superoxyde dismutase 1 (*OmSOD1*). Il s'agit de la première délétion d'un gène par recombinaison homologue chez un champignon mycorhizien. Nous démontrons que l'absence d'*OmSOD1* cause un déséquilibre dans l'homéostasie rédox et un changement dans le dialogue entre le champignon et sa plante hôte.
La deuxième approche a été basée sur la complémentation fonctionnelle d'un mutant de levure en utilisant une banque d'ADNc d'*O. maius*. Nous décrivons les premiers transporteurs d'un champignon mycorhizien éricoïde capables de conférer la tolérance au Zn dans des levures. Deux gènes ont été isolés et nommés OmCDF et OmFET. L'expression hétérologue de ces deux gènes dans différents mutants de levure a permis de conférer la tolérance au Zn. De plus, *OmCDF* a également permis de conférer la tolérance au Co. Nos données suggèrent que OmCDF est un transporteur de Zn responsable du transfert du Zn cytoplasmique vers le réticulum endoplasmique, tandis que l'expression d'OmFET pourrait neutraliser la toxicité engendrée par le Zn en augmentant le contenu du Fe dans la cellule.
La troisième approche a concerné le criblage d'une collection de mutants aléatoires d'*O. maius* sur Zn, Cd et ménadione. Nous décrivons la caractérisation d'un mutant dans le gène *nmr*. Dans ce mutant, une diminution de la teneur en glutamine et asparagine, ainsi qu'une réduction de l'activité de la glutamine synthétase ont été enregistrées. Les liens possibles entre la tolérance au stress oxydatif et le métabolisme azoté sont discutés.

Mots clés : champignon mycorhizien éricoïde, mécanismes de tolérance, métaux lourds, stress oxydatif.