

Chapter 2

Toxic Metal/Metalloid Tolerance in Fungi—A Biotechnology-Oriented Approach

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Abstract This review aims at the biotechnological evaluation of the wealth of data accumulated in the last decade on the molecular background of the toxic metal/metalloid tolerance of fungi. Yeast-based models are highly applicable when metal/metalloid transport and compartmentalization processes are mapped in other fungal species or higher eukaryotes but this approach has limitations, which necessitates further fungal models evolutionarily closer to heavy metal exposed fungal taxons. In terms of biotechnology, the most promising targets in the genetic engineering of metal/metalloid tolerant fungi include (i) increased secretion of extracellular metal chelators, (ii) elimination of metal transporters facilitating the uptake of toxic metals/metalloids, (iii) overexpression of transporters pumping metals and/or their complexes out of the cells or into cellular organelles, (iv) overproduction of intracellular metal chelators, (v) overproduction of elements of the antioxidative defense system, (vi) genetic modification of the regulatory network of metal/metalloid stress defense, and (vii) interfering with the metal/metalloid-dependent initialization of apoptotic cell death. Owing to the wide-spread application of robust ‘-omics’ technologies, the biotechnologically exploitable data including potential future targets for genetic manipulation are accumulating fast. In contrast, today’s genetic modifications often result in unforeseeable or even paradox phenotypes in this field, which clearly indicates that a deeper understanding of the underlying molecular mechanisms of fungal toxic metal/metalloid tolerance is needed to improve the biotechnological performance of the genetically modified strains.

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Introduction

Metal pollutants are released into the environment in many ways at potentially harmful levels (Avery 2001). In addition to vast industrial and agricultural discharges, different metal compounds are also used to treat severe human diseases like promyelocytic leukemia {As(III)} and leishmaniasis {Sb(V)} (Tamás et al. 2005). In some areas, the origin of the heavy metals and/or metalloids appearing in the food chain is geological rather than anthropogenic (like the As contamination of drinking water). Because some toxic metals and metalloids are widely distributed in nature microorganisms developed (and are developing!) quite effective strategies to cope with the harmful consequences of metal/metalloid exposures (Tamás et al. 2005).

Today's knowledge on the molecular background of metal/metalloid toxicity in eukaryotes arises in great part from extensive genome, transcriptome, deletome, proteome, interactome and metabolome analyses having been done in baker's yeast cultures. *Saccharomyces cerevisiae* is an excellent model organism to address important biological questions because the available molecular biological, genetic and bioinformatic tools are unprecedentedly sophisticated and versatile with this hemiascomycete. As shown by numerous examples, knowledge obtained from yeast-based models, e.g. on the mechanism of action of and the tolerance against toxic metals, can be transferred with high efficiency to higher eukaryotes including humans (Tamás et al. 2005; Wysocki and Tamás 2010).

On the other hand, the wide evolutionary distances between hemiascomycetes and other major fungal taxons warn us that the applicability of yeast-based models to map metabolic and regulatory pathways in other fungi (even in ascomycetes!) may have some limitations (Miskei et al. 2009). In fact, some well-characterized metal/metalloid stress response pathways in baker's yeast, e.g. the Yap8p-dependent regulation of As(III) stress response, seem to be clearly hemiascomycete specific and, therefore, are not exploitable in other fungi (Wysocki and Tamás 2010). Suitable molecular tools and coordinated community efforts are eagerly needed to set up additional fungal models evolutionarily closer to the heavy metal exposed fungal taxons like the mycorrhizal species (Hildebrandt et al. 2007; González-Guerrero et al. 2009).

Considering the wealth of information accumulated on the toxic metal/metalloid tolerances of yeasts and filamentous fungi in the last decade, an applied microbiology/biotechnology-oriented researcher may feel a bit puzzled when she/he aims at the production of fungal strains with a significantly augmented toxic metal/metalloid tolerance and/or with enhanced metal/metalloid biosorption and bioaccumulation properties. For example, the overlap between the genome-wide screens for genes contributing to toxic metal/metalloid tolerance may be as low as 10–20% although the gene groups coming from different laboratories and surveys complement nicely each other in terms of physiological functions (Thorsen et al. 2009). Because excellent reviews are available in this field, which are summarizing today's yeast-based knowledge on toxic metals and metalloids in an easy-to-read and easy-to-understand way (Tamás et al. 2005; Wysocki and Tamás 2010), the focus of this

review is placed on the possible future targets of the genetic engineering of toxic metal/metalloid tolerant fungi. Moreover, the chapter incorporates the most relevant physiological and genetic data gained in toxic metal/metalloid exposed filamentous fungi as well as in yeast and filamentous fungus cultures exposed to essential micronutrient metals like Fe, Cu or Zn.

It is the hope of the author that a biotechnology-oriented evaluation and systematization of our current understanding of fungal metal/metalloid tolerance would help us to develop new, environmentally friendly and economically feasible technologies in the remediation of heavy metal contaminated soil (Gadd 2000, 2010; Schützendübel and Polle 2002; Gaur and Adholeya 2004; Khan 2005; Lebeau et al. 2008; Marques et al. 2009; Bothe et al. 2010; Hong-Bo et al. 2010; Purakayastha and Chhonkar 2010; Wu et al. 2010) and water (Baldrian 2003; Agrawal et al. 2006; Swami and Buddhi 2006; Wang and Chen 2006; Pan et al. 2009, More et al. 2010; Sankaran et al. 2010), in bioleaching of heavy metals from preservative-treated wood (Mai et al. 2004; Sierra-Alvarez 2007, 2009), and in the mining and recovery of metals (Mulligan et al. 2004; Fujii and Fukunaga 2008; Kuroda and Ueda 2010; Simate et al. 2010). Fungal toxic metal/metalloid resistance genes heterologously expressed in plants (Mejäre and Bülow 2001; Song et al. 2003; Wawrzyński et al. 2006; Guo et al. 2008) may enhance the efficiency of the available phytoremediation or phytomining technologies (Lasat 2002; Chaney et al. 2007; Sheoran et al. 2009).

Considering the structure of this review, after the presentation and discussion of the major elements of fungal metal/metalloid stress defense systems, readers' attention is called to some promising future targets and genetic tools to increase the metal/metalloid tolerance of fungi.

First Line of Defense: Extracellular Chelation and Binding to Cell Wall Constituents

Metal chelation by small molecular mass metabolites, peptides and proteins is a crucially important element of almost all metal/metalloid detoxification processes (Tamás et al. 2005; González-Guerrero et al. 2009; Wysocki and Tamás 2010) and, hence, the significance of extracellular and cytosolic chelation reactions cannot be overestimated. Glutathione (GSH) secretion is a very important element of the GSH-homeostasis in yeast under different environmental conditions (Perrone et al. 2005), and it is sensible that yeast cells intensify GSH-secretion under As(III)-exposures to relieve the intracellular detoxification pathways (Wysocki and Tamás 2010).

Oxalate secretion is well-documented in both brown-rot and white-rot fungi, and this process seems to be stimulated under Cu(II) and Cd(II) stress (Clausen and Green 2003; Jarosz-Wilkolazka et al. 2006). The bulk formation of water-insoluble metal-oxalate crystals is undoubtedly an efficient way to prevent toxic metal ions entering fungal cells (Jarosz-Wilkolazka and Gadd 2003). In addition, oxalate is

primarily important to maintain the lignolytic system of white rot basidiomycetes (Schlosser and Höfer 2002).

A wide range of fungi has been reported to produce extracellular mucilaginous materials (ECMM or “emulsifier”) with excellent toxic metal binding capabilities. As demonstrated by Paraszkievicz et al. (2007, 2009, 2010), Ni(II) {unlike Cu(II), Pb(II) and Zn(II)} did not trigger ECMM production by *Curvularia lunata*. These authors reported the saturation of cellular fatty acids, which is clearly attributable to Ni(II)-initiated lipid peroxidation processes. Importantly, the pullulan production by *Aureobasidium pullulans* was positively affected by Ni(II) and Cd(II) exposures (Breierová et al. 2004), and pullulan increased the Cd(II) tolerance of this industrially important species (Čertík et al. 2005). Vesentini et al. (2006) also reported elevated ratio of ECMM in the biomass in Cu(II) exposed *Trametes versicolor* and *Gloeophyllum trabeum* cultures.

Isolates of the arbuscular mycorrhizal fungi *Glomus* and *Gigaspora* species produce a soil glycoprotein called glomalin (Wright et al. 1996), which possesses a remarkable capability to sequester Cu(II) (González-Chávez et al. 2004; Cornejo et al. 2008; Ferrol et al. 2009). Glomalin is located mainly in the cell wall (Purin and Rilling 2008; Ferrol et al. 2009). Besides glomalin, other cell wall polymers like chitin and melanin can also take part in metal biosorption (González-Guerrero et al. 2009). As demonstrated by Lanfranco et al. (2002a, 2004), Zn(II) exposures affected chitin synthase gene expression patterns and chitin deposition in an ericoid mycorrhizal fungus.

Although decreasing the bioavailability of the toxic metals/metalloids through extracellular complexation, precipitation, and binding to cell wall constituents represents a reasonable and straightforward strategy in strain developments the genetic tools to approach this aim are still in a premature stage. The lack of yeast systems suitably modeling extracellular toxic metal/metalloid detoxification pathways obviously represents a major handicap especially when it is compared to the abundance of models and information available on the organization and regulation of intracellular detoxification pathways.

Second Line of Defense: Transport, Intracellular Chelation and Compartmentalization

Heavy metals enter cells through channels and transporters, which normally facilitate the uptake of essential transition metal micronutrients like Fe, Mn and Zn, anions including phosphate and sulphate as well as sugars (glucose) and sugar derivatives (glycerol) (Tamás et al. 2005; Wysocki and Tamás 2010). In theory, one of the most simple and most effective way to keep off toxic metals/metalloids outside the cell is to eliminate the channel or transporter responsible for the uptake of a given toxic metal/metalloid ion. Unfortunately, these ions may be channeled through multiple transporters into the cytoplasm and, aggravating the situation, the absence of even one of these transport routes may disturb the normal metabolism of the cells.

The elimination of the following plasma membrane channels and transporters has been demonstrated to confer metal tolerance to metal/metalloid exposed *S. cerevisiae* cells (Tamás et al. 2005; Wysocki and Tamás 2010):

- As(V): Pho84p high-affinity and Pho87p low-affinity phosphate transporters (Bun-ya et al. 1996),
- As(III) and Sb(III): Fps1p aquaglyceroporin channel (Wysocki et al. 2001),
- Cd(II): Zrt1p Zn(II) and Smf1p and Smf2p Mn(II) transporters (Gomes et al. 2002; Gitan et al. 2003; Ruotolo et al. 2008),
- Cr(VI) and Se(VI): Sul1p and Sul2p sulphate transporters (Cherest et al. 1997).

Importantly, yeast cells lacking either Pho86p, which is required in trafficking Pho84p from the ER to the cytoplasmic membrane, or Gtr1p, a cytoplasmic GTP binding protein, a regulator of phosphate transport through Pho84p, displayed As(V)-tolerant phenotypes (Bun-ya et al. 1992, 1996; Yompakdee et al. 1996). Furthermore, the elimination of *BSD2* encoding an ER protein trafficking Smf1p and Smf2p transporters to the vacuoles for degradation resulted in Cd(II) and Cu(II) hypersensitivities (Liu et al. 1997; Liu and Culotta 1999). All these observations suggest that intracellular protein trafficking systems represent relevant and promising targets for engineering new fungal strains with an altered toxic metal/metalloid tolerance.

The heavy metal influx through micronutrient metal transporters can be blocked by supplementing the culture media with high concentrations of the essential ions like Zn(II), which leads to the elimination of the zinc-transporter Zrt1p (Gitan et al. 1998, 2003). In the biotechnologist's point of view, the variation of the metal content of the culture media to hinder toxic metal/metalloid uptake is always an attractive alternative to any kind of genetic manipulations of the transport processes themselves.

Nevertheless, overexpression of transporters pumping toxic metals/metalloids and/or their chelates out of the cells or into subcellular organelles (primarily into the vacuoles) may represent a distinguished tool in the genetic engineering of metal resistant fungal strains. Potential targets include:

- As(III): Acr3p plasma membrane transporter (Wysocki et al. 1997) and, somewhat paradoxically, Fps1p aquaglyceroporin channel, which is likely to perform As(III) and Sb(III) export as well (Maciaszczyk-Dziubinska et al. 2010),
- Cd(II) and Cu(II): Pca1p plasma membrane P-type ATPase (Adle et al. 2007),
- Co(II), Rh(II) and Zn(II): Cot1p vacuolar Zn(II) transporter (Conklin et al. 1992; MacDiarmid et al. 2000),
- Fe(II) and Mn(II): Ccc1p vacuolar transporter (Li et al. 2001),
- Se(IV): Ssu1p plasma membrane sulphite pump (Pinson et al. 2000),
- Zn(II) and Co(II): Zrc1p vacuolar membrane Zn(II) transporter (Conklin et al. 1994),
- GSH complexes of Cd(II), As(III), Hg(II), Pb(II): Ycf1p ATP-binding cassette family vacuolar GSH S-conjugate transporter, a strong induction of which can be achieved by overexpressing Yap1p, the master regulator transcription factor

of oxidative stress response (Wemmie et al. 1994; Sharma et al. 2002; Song et al. 2003); Yor1p plasma membrane ABC transporter (Nagy et al. 2006),

- phytochelatin complexes of Cd(II): HMT1 vacuolar membrane transporter of ATP-binding cassette-type (*S. pombe*; Ortiz et al. 1995), although HMT1 is likely to be a GSH S-conjugate transporter in *S. pombe* according to most recent data published by Prévéral et al. (2009).

Importantly, vacuolar Zn(II) (GintZnT1; González-Guerrero et al. 2005) and Cd(II)/Cu(II) (GintABC1; González-Guerrero et al. 2009, 2010a) transporters have been characterized in the endomycorrhizal fungus *Glomus intraradices* with high homology and functional orthology to well-characterized baker's yeast proteins, which clearly demonstrates the applicability of yeast-based heavy metal sequestration models in this case. Not surprisingly, the expression of *GintABC1* was up-regulated when the fungus was exposed to Cd(II) and Cu(II) (González-Guerrero et al. 2010a).

In terms of biotechnology, overexpression of fungal metal/metalloid transporters in transgenic plants is a viable option when new-type phytoremediators with increased metal/metalloid bioaccumulation and tolerance are engineered. A notable example comes from the work published by Song et al. (2003) when the Ycf1p transporter of *S. pombe* was heterologously expressed in *Arabidopsis thaliana* resulting in increased Cd(II) and Pb(II) tolerances.

One may tackle the cytosolic metal toxicity problem solely by increasing the biosynthesis of metal chelators. GSH is a key player in both heavy metal tolerance and oxidative stress defense (Wu and Moye-Rowley 1994; Stephen and Jamieson 1997; Westwater et al. 2002; Pócsi et al. 2004; Mendoza-Cózatl et al. 2005; Tamás et al. 2005; Hegedűs et al. 2007; Wysocki and Tamás 2010), and its overexpression increases the toxic metal/metalloid tolerance. When yeast γ -glutamylcysteine synthetase *GSH1* and garlic phytochelatin synthase *AsPCS1* were expressed either alone or simultaneously in *Arabidopsis thaliana* the transgenic plants accumulated and tolerated Cd and As remarkably well (Guo et al. 2008). Moreover, transgenic tobacco (*Nicotiana tabacum* cv. LA Burley 21) plants harboring *E. coli* serine acetyltransferase (Cys biosynthesis), *E. coli* γ -glutamylcysteine synthetase (GSH biosynthesis) and *S. pombe* phytochelatin synthase genes showed an increased non-protein thiol production and an elevated Cd(II) accumulation in the roots but not in the shoots (Wawrzyński et al. 2006). It is worth noting that recombinant GSH-overproducing yeast strains have also been engineered using self-cloning modules containing the *GSH1* gene (*S. cerevisiae*; Wang et al. 2007, 2009) or an intracellular expression vector with *GSH1* and *GSH2* biosynthesis genes (*Pichia pastoris*; Fei et al. 2009). Intracellular GSH levels can also be elevated considerably in baker's yeast by overexpressing the Hgt1p GSH-transporter (Srikanth et al. 2005) but resulted in some GSH-induced cell toxicity (Srikanth et al. 2005), indicating the limitation of this approach.

Considering GSH turnover, vacuolar γ -glutamyltranspeptidase and aminopeptidase are needed to recycle GSH-derived amino acids into the cytoplasm (Pócsi et al. 2004). Interestingly, a yeast strain with *LAP4* mutation accumulated three

times more Cd(II) than the control strain with an unexpected decrease in the Cd(II)-elicited oxidative stress (Adamis et al. 2009)—an observation with the potential of biotechnological application.

Overexpression of phytochelatins or even implant the phytochelatin biosynthetic pathway into fungi without any capability to synthesize this kind of highly efficient chelating molecules could improve profoundly the metal/metalloid tolerance of the recipient fungal strains. In fact, heterologous expression of either *S. pombe*, plant, red alga or nematode phytochelatin synthases conferred Cd(II), Cu(II), As(III) and Sb(III) resistance to the yeast *S. cerevisiae* (Clemens et al. 1999; Vatamaniuk et al. 2001; Wysocki et al. 2003; Rea et al. 2004; Ramos et al. 2007; Osaki et al. 2008). It were definitely worth seeing the heavy metal tolerance of good GSH-producer ectomycorrhizal fungi like *Paxillus involutus* (Ott et al. 2002; Courbot et al. 2004; Hegedűs et al. 2007) after the phytochelatin synthase biosynthetic capability had been implanted!

Interestingly, *S. pombe* deposits Cd(II) in the form of vacuolar high molecular mass phytochelatin-Cd(II) aggregates with CdS crystallites in their cores (Dameron et al. 1989; Mendoza-Cózatl et al. 2005). These semiconductor nanoparticles can be used in the fabrication of diodes (Kowshik et al. 2002), and genetically engineered *E. coli* cell lines (Kang et al. 2008; Chen et al. 2009) as well as *Lactobacillus* sp. and *S. cerevisiae* based systems (Prasad and Jha 2010) are now also available to produce CdS nanocrystals. In terms of metal/metalloid detoxification, S²⁻-overproduction seems to be beneficial in yeast in general. This can be achieved in several ways including the construction of *MET2* (encoding homoserine *O*-acetyltransferase) or *MET17/MET15/MET25* (encoding *O*-acetylserine and *O*-acetylhomoserine sulfhydrylase) mutations (Ono et al. 1991). Alternatively, *via* the ubiquitination of the Met4p transcriptional regulator by overexpressed Cdc34p ubiquitin-conjugating enzyme could results in a reduced expression of *MET17/MET15/MET25* (Hwang et al. 2007).

Metallothioneins are low molecular mass metal chelator proteins with high affinity towards Cu(II), Zn(II) and Cd(II) (Ecker et al. 1986; Borrelly et al. 2002; Zhang et al. 2003; Kumar et al. 2005; Wysocki and Tamás 2010). *S. cerevisiae* may contain tandem repeats of the *CUP1* metallothionein gene, and the number of the gene copies correlates with the Cu(II) and Zn(II) binding capacities and the Cu(II)-tolerance of the yeast cells (Stroobants et al. 2009). Heterologously expressed yeast metallothionein Cup1p promoted successfully the uptake of Cu(II) from contaminated soils by transgenic tobacco plants (Thomas et al. 2003). Furthermore, metallothionein PiMT1 found in *P. involutus* complemented the Cu(II) and Cd(II) hypersensitivity of metallothionein deficient yeast strains and even increased the Cu(II) tolerance of the ectomycorrhizal fungus *Hebeloma cylindrosporum* (Bellion et al. 2007). Most recently, the functional characterization of *H. cylindrosporum* metallothioneins HcMT1 and HcMT2 took place (Ramesh et al. 2009). Considering the fact that metallothioneins of taxonomically distant arbuscular mycorrhizal fungi, *e.g.* GmarMT1 (*Gigaspora margarita*; Lanfranco et al. 2002b) and GintMT1 (*G. intraradices*; González-Guerrero et al. 2007), share common features with yeast metallothioneins, *e.g.* Cu(II) and Cd(II) sequestering capabilities, we can conclude

that yeast-based metal tolerance models are highly applicable for taxonomically distant fungal species in this case.

Intriguingly, one of the centerpieces of the antioxidative defense systems of fungi, Cu,Zn-superoxide dismutase (Cu/Zn-SOD), may also play a pivotal role in buffering intracellular Cu(II) concentrations *via* incorporating superfluous (and redox active!) free Cu(II) under both aerobic and anaerobic conditions (Culotta et al. 1995; Avery 2001). Genetic engineers may take advantage of the Cu(II) {and Zn(II)?} binding potential of Cu,Zn-superoxide dismutases in the future when Cu(II) and Zn(II) tolerant fungal strains are required for different technological purposes like in water or soil bioremediation programs (Vallino et al. 2009; Villegas et al. 2009).

Similar to copper, the redox active iron is also an essential metal and needs a proper uptake, intracellular transport and storage to avoid the deleterious consequences of the appearance of free iron in either the cytosol or the cell organelles. In filamentous fungi, intracellular siderophores like ferriicrocin and hydroxyferriicrocin have been reported to keep excess iron in a thermodynamically inert state (Eisendle et al. 2003, 2006; Schrettl et al. 2007, 2008; Johnson 2008). The iron regulon including the siderophore biosynthetic pathways are tightly and negatively regulated by the GATA factor SreA in *Aspergillus fumigatus* (Schrettl et al. 2008). The complex forming ability of hexadentate hydroxamate fungal siderophores with heavy metals other than Fe(III) is relatively weak (Enyedy et al. 2004; Farkas et al. 2008) and, as a consequence, their potential in heavy metal detoxification technologies does not seem to be significant. On the other hand, these Fe(III)-complexing compounds reduce the free Fe-dependent steps in the pathogenesis of atherosclerosis *in vitro* and one of them, desferricoprogen is absorbed effectively in the gastrointestinal tract of rats (Pócsi et al. 2008). These observations point to a novel role of fungal iron chelators, *e.g.* in the field of food biotechnology in the development of functional foods and food additives (Pócsi et al. 2008; Tóth et al. 2009).

In baker's yeast, iron homeostasis is balanced by the Aft1p and Aft2p transcriptional activators (Yamaguchi-Iwai et al. 1995; Blaiseau et al. 2001; Johnson 2008). The yeast frataxin Yfh1p may play a crucial role in the coordination of mitochondrial iron use in basically important mitochondrial metabolic processes like heme biosynthesis and [Fe-S] cluster assembly and stabilization. Yeast strains defective in frataxin synthesis represent applicable models of a well-known human iron metabolic disorder called Friedreich's ataxia (De Freitas et al. 2003). The self-assembly of yeast frataxin is iron-dependent and is leading to multimers (up to 60 subunits), which can sequester >3,000 atoms of iron (Adamec et al. 2000). In addition, it is highly reasonable to assume that Yfh1p is an iron chaperone with primary function in mitochondrial iron detoxification (Bulteau et al. 2004; Gakh et al. 2006, 2008). In good accordance with this, the expression of the human iron storage protein ferritin in frataxin-deficient yeast cells rescued the majority of the mitochondrial functions deteriorated in the absence of Yfh1p and extended the lifespan of the mutant (Campanella et al. 2004; Desmyter et al. 2008). However, frataxin overexpression in yeast affected iron-dependent metabolic processes ambiguously (increased heme synthesis *vs.* defective [Fe-S] cluster assembly/stability; Seguin et al. 2009), but did

not alter the soluble-to-insoluble iron ratio in mitochondria (Seguin et al. 2010). These observations necessitate further studies to clarify the exact physiological functions of these important mitochondrial proteins, and their potential biotechnological application.

Vacuoles represent the primary sites of intracellular metal/metalloid sequestration and storage in fungi (Tamás et al. 2005; Wysocki and Tamás 2010). It is noteworthy that under the depletion of essential metals vacuolar metal deposits can be mobilized including Zn(II) (Zrt3p, MacDiarmid et al. 2000), Cu(II) (Ctr2p, Rees et al. 2004) and Fe(II) (Smf3p; Portnoy et al. 2000; Fet5p-Fth1p complex, Urbanowski and Piper 1999). Due to the high turnover of redox active essential metals, the redox milieu of the vacuoles has to be fairly stable to avoid the generation of highly fragmented and damaged organelles (Corson et al. 1999).

As discussed by Ferrol et al. (2009), the vacuolar metal binding activity shows an uneven distribution in arbuscular mycorrhizal fungi mycelia with clear-cut maxima in the intraradical vesicles and extraradical spores (Weiersbye et al. 1999; González-Guerrero et al. 2008; Orłowska et al. 2008). In a spectacular study performed by González-Guerrero et al. (2008), high Cu(II)-accumulating vacuoles were clearly concentrated in the blue-green colored spores of *G. intraradices*.

Third Line of Defense: The Antioxidative Defense System

Fungi exposed to toxic metal/metalloid stress commonly face oxidative cell injuries caused by reactive oxygen species (Avery 2001). Fungal cells possess a wide array of antioxidants to cope with different kinds of oxidative stress. For example, GSH-independent and GSH-dependent enzyme activities are able to neutralize reactive oxygen species with remarkable efficiency (Pócsi et al. 2004).

As mentioned above, Cu/Zn-superoxide dismutases buffer redox active Cu(II) levels within the cytosol (Culotta et al. 1995) but also catalyze superoxide \rightarrow peroxide + O₂ conversions. The elimination of this type of enzyme results in a metal sensitive phenotype in fungi (Sumner et al. 2005), elevation in the intracellular “free iron” concentrations (Srinivasan et al. 2000), fragmenting vacuoles (Corson et al. 1999) and, as a consequence, reorganization of the iron metabolic network (De Freitas et al. 2000). As demonstrated by González-Guerrero et al. (2010b), the endomycorrhizal fungus *G. intraradices* also has a Cu(II)-inducible Cu,Zn-superoxide dismutase (GintSOD1). Interestingly, the Zn(II)-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn releases a Cu,ZnSOD into the extracellular environment, which may contribute to the metal tolerance of the plant symbiont as well (Vallino et al. 2009). It is reasonable to assume that the overexpression of Cu/Zn-superoxide dismutase in fungal cells would provide them with an improved metal and oxidative stress tolerance. But increased oxidative stress tolerance and chronological life span were only observable in yeast cells when Sod1p was co-expressed with the Cu(II)-chaperone Ccs1p (Brown et al. 2004) and the culture medium was supplemented with high Cu(II) concentrations (Harris et al. 2005). In the absence of

either Ccs1p overexpression or high-dose Cu(II)-supplementation the cells showed the symptoms of emerging oxidative stress and a shortened chronological life span (Harris et al. 2005).

For the biotechnologist, one of the most attractive options to improve the performance of Cu/Zn-superoxide dismutases in any engineered fungi is the replacement of the wild-type enzyme with a Cu/Zn-superoxide dismutase with superior enzymological parameters. For example, the Cu/Zn-superoxide dismutase cloned and characterized in the deep-sea yeast *Cryptococcus liquefaciens* strain N6 possessed four-fold higher activity than baker's yeast's Sod1p and was functional in *S. cerevisiae* (Kanamasa et al. 2007).

The overexpression of the mitochondrial flavohemoglobin 2, which has a role in nitric oxide detoxification, resulted in an unexpected H₂O₂-hypersensitive phenotype in *Aspergillus oryzae* (Zhou et al. 2010). This observation warns us that the overproduction of any antioxidants in fungal cells may turn up unforeseeable or even disadvantageous physiological consequences especially when the antioxidants are not well-characterized or when their overproduction is not controlled satisfactorily.

Paradoxically, a tight control of glutathione reductase activity may result in a Cr(VI)-tolerant phenotype in fungi because this central element of the GHS-dependent antioxidative defense system catalyzes Cr(VI)→Cr(V) conversion giving rise to harmful redox species and cycling reactions in yeasts (Pesti et al. 2002; Gazdag et al. 2003; Koós et al. 2008; Poljšak et al. 2010).

It is worth mentioning that heterologous expression of *E. coli* GSH biosynthetic enzymes in Indian mustard (*Brassica juncea* L.) plants increased the toxic metal/metalloid tolerance of the plant (Zhu et al. 1999a, b; Reisinger et al. 2008)—a promising strategy, which might be exploitable in fungi as well.

Antioxidants, such as ascorbic acid can reduce transition metals giving these compounds a pro-oxidant character (Poljšak et al. 2005). Therefore any overload of fungal cells with antioxidants (e.g. Tempol, Trolox, melatonin) might exacerbate oxidative cell damages in the presence of redox active metals/metalloids (Lewinska and Bartosz 2007). In accordance with these observations, deletion of sulphite reductases (*MET10* and *MET5*) in the sulphate assimilation pathway, which is basically important in the *de novo* synthesis of S-containing amino acids and the antioxidant GSH, prevents Te(III)→Te(0) conversion and results in an enhanced metalloid tolerance in baker's yeast (Wysocki and Tamás 2010). All these observations indicate that any modulation of the antioxidative defense systems of fungi to increase metal/metalloid tolerance should be done by extreme care because of the unforeseeable impacts on the pro-oxidant/antioxidant status of the cells.

Another option to gain metal/metalloid tolerant fungal strains is the overexpression of elements of the signal transduction and regulatory pathways, which are normally operating in metal/metalloid stress exposed wild-type strains (Avery 2001; Haugen et al. 2004; Tamás et al. 2005; Rodrigues-Pousada et al. 2010; Wysocki and Tamás 2010).

The master regulator, which orchestrates metal/metalloid stress responses in baker's yeast {e.g. under Cd(II), As(III), Sb(III), Se(III) and Hg exposures;

(Wysocki and Tamás 2010)} is the bZIP-type transcriptional factor Yap1p. As demonstrated by Wemmie et al. (1994), wild type yeast cells harboring multiple copies of *YAP1* possessed an increased Cd(II)-tolerance. Another bZIP-type factor, Yap2p, suppressed the Cd(II)-sensitive phenotype of a *YAP1* disruptant although the *YAP2* disruptant itself was not characterized with any increased Cd(II)-sensitivity (Hirata et al. 1994). According to Azevedo et al. (2007), Yap1p and Yap2p transcription factors share a common Cd(II)-sensing domain. Considering other bZIPs, Yap5p is involved in the regulation of the Fe-homeostasis *via* the regulation of *CCC1* encoding the vacuolar iron transporter Ccc1p (Li et al. 2008), and Yap8p plays a pivotal role in the regulation of As(III)-detoxification (Haugen et al. 2004; Wysocki et al. 2004).

The appearance of high quantities of damaged, non-functional, mistranslated or even aggregated proteins is a common symptom of metal/metalloid toxicity (Holland et al. 2007; Medicherla and Goldberg 2008). Proteasomes are in the first line of the removal and degradation of such proteins, and this activity is regulated positively by the transcriptional factor Rpn4p (Haugen et al. 2004; Thorsen et al. 2007, 2009; Medicherla and Goldberg 2008). One may speculate that a constitutively high-level expression or even the overexpression of Rpn4p would lead to the development of metal/metalloid tolerant fungal strains but, again, the outcome of such genetic modifications may be quite different. As demonstrated by Wang et al. (2010), both the availability and the proteasomal degradation of Rpn4p are equally important factors to maintain cell viability under various types of stress conditions!

The mitogen-activated protein kinase Hog1p also plays an indispensable role in metal/metalloid stress defense in yeasts (Wysocki and Tamás 2010). The Hog1p of *S. cerevisiae* downregulates As(III) transport activity *via* Fps1p (Thorsen et al. 2006; Dilda et al. 2008), controls cell cycle progression (Migdal et al. 2008) meanwhile the orthologous *S. pombe* (Sty1/Spc1) and *C. albicans* (Ca-Hog1) kinases are transported to the nuclei upon Cd(II) and As(III) exposures and reprogram global transcription (Enjalbert et al. 2006; Kennedy et al. 2008). On the other hand, the hyperactivation of Hog1p is deleterious for yeasts (Wurgler-Murphy et al. 1997; Warmka et al. 2001).

Not surprisingly, the major nutrient-responsive signal transduction pathways TOR (target of rapamycin) and PKA (protein kinase A) also contribute to the regulation of general stress responses in yeast (Wysocki and Tamás 2010). Under nutrient abundance, these pathways stimulate physiological processes maintaining a high growth rate including the down-regulation of stress defense systems. Under nutrient limitation or toxic metal/metalloid exposures, Msn2p/Msn4p transcription factors are activated and they stimulate the expression of a wide array of antioxidant enzymes (Gasch et al. 2000; Hosiner et al. 2009). Interestingly, the elimination of *MSN2* and *MSN4* resulted in an As(III)-tolerant phenotype in *S. cerevisiae*, which might be indicative of an exaggerated expression of stress-defense genes (Hosiner et al. 2009). Another transcription factor, Sfp1p is removed from DNA when TOR activity is inhibited by metal/metalloid stress and this detachment leads to the downregulation of ribosomal protein synthesis (Marion et al. 2004; Hosiner et al. 2009). Unexpectedly, deletion of *SFP1* resulted in an increased As(III)-tolerance (Hosiner et al. 2009).

Metal/metalloid stress response regulatory elements do not work independently of each other (Salin et al. 2008). For example, Yap1p (redox homeostasis), Rpn4p (protein degradation), Hsf1p (protein folding), Pdr1p, Pdr3p, Yrr1p (multidrug resistance), Aft2 (iron homeostasis) and Cin5p (osmotic stress tolerance) transcription factors join in a highly interconnected regulatory network including transcriptional loops to orchestrate Se(III) stress response in the most efficient way (Salin et al. 2008). Obviously, the elimination or modification of any element of the network may trigger unpredictable physiological changes and the appearance of unforeseeable phenotypes, which makes the genetic engineering of the regulatory network itself to develop metal/metalloid tolerant mutants risky, and still largely empirical with some unexpected consequences.

Screening for Future Targets to Engineer Heavy Metal Tolerant Fungi

Genome, transcriptome, deletome, proteome, metabolome and interactome analyses have been performed and are in progress to learn how different fungi respond to versatile toxic metal/metalloid exposures. To become familiar with the elements and the regulation of the metal/metalloid stress response networks may provide us with suitable tools to augment the metal/metalloid tolerance of selected fungi with potential applications in a wide spectrum of environmental technologies. This subchapter summarizes the most promising pieces of information extracted from the abundant experimental data having been generated in the last decade using robust “-omics” techniques.

In their substantial work, Jin et al. (2008) compared the transcriptome profiles of baker's yeast exposed to Ag(I), As(III), Cd(II), Cr(VI), Co(II), Hg(II) and Zn(II), and they found that the chemical properties of the metals/metalloids basically determined the pattern of the genome-level transcriptional response. Not surprisingly, principal component analysis and hierarchical clustering of the expression data by treatment conditions grouped Ag(I)-Zn(II), Cd(II)-Hg(II) and As(III)-Cu(II) metal/metalloid pairs close together meanwhile Cr(VI) was separated from other metals—a valuable piece of information when fungal strains with multiple metal resistances should be developed. Jin et al. (2008) managed to identify the group of CRM (Common Metal Responsive) genes, which may provide us with the possibility to construct fungal strains with a general metal/metalloid tolerance! Overexpressions of genes encoding *e.g.* polyamine and iron transporters, proteins maintaining ion homeostasis and iron transport as well as elements of the antioxidant defense system, or elimination of genes encoding *e.g.* protein kinase subunits and transition metal and carbohydrate transporters (Jin et al. 2008) can be among the most promising targets of future genetic engineering technologies. On the other hand, deletome analysis performed concomitantly by Jin et al. (2008) indicated that the metal-resistance and metal-sensitivity genes were basically metal-specific. As a consequence, the

metal/metalloid-responsive transcriptome and deletome datasets shared as few as 22 genes in overlapping regions including *CYS3* {cystathionine γ -lyase; essential for survival in the presence of As(III), Cd(II) and Cu(II)}, *ADH1* {alcohol dehydrogenase; important in As(III) and Cu(II) tolerances} and *RNR1* {ribonucleotide-diphosphate reductase; with pivotal importance in Cd(II) and Cr(IV) tolerances}. Furthermore, sulphur amino acid transport and biosynthesis were essential under As(III), Cd(II), Cr(VI) and Cu(II) stress conditions.

In accordance with and partly supplementary to the observations of Jin et al. (2008), Ruotolo et al. (2008) demonstrated by deletome and interactome analyses that tolerance against both Cd(II) and Ni(II) exposures relied on the concerted action of several functional subnetworks including proteasome, vacuolar fusion, cell wall integrity pathway, v-ATPase assembly/regulation, essential ion homeostasis, ERG pathway, nuclear pore complex, Ccr4p and other mRNA processing enzymes and chromatin remodeling. Another yeast deletome analysis performed by Thorsen et al. (2009) with As(III) and Cd(II) indicated that metal/metalloid tolerance required sulphur and glutathione biosynthesis, environmental sensing, mRNA synthesis and transcription as well as vacuolar/endosomal transport and sorting. The importance of the sulphur assimilation pathway in Cd(II)-exposed *S. cerevisiae* cells was also emphasized by several other authors performing transcriptome (Momose and Iwahashi 2001; Haugen et al. 2004) and proteome (Vido et al. 2001) studies. Sulphur starvation caused by Cr(VI) exposures was also demonstrated in baker's yeast by Pereira et al. (2008) at transcriptome level, and the channeling of sulphur towards GSH synthesis by stimulating the transcription of sulphur-poor isoenzymes (the sulphur-sparing response) was shown in *S. cerevisiae* treated with Cd(II) and Cr(VI) (Fauchon et al. 2002; Pereira et al. 2008). Overlapping oxidative stress responses were also recorded in the yeast *Hansenula polymorpha* exposed to Cu(II) or V(V) (Mannazzu et al. 2000).

Further interesting metal-specific stress-responsive cell biological, metabolic and regulatory pathways, which are necessary to gain metal/metalloid tolerance in *S. cerevisiae*, are also presented here, and the data are grouped according to the toxic metals/metalloids:

- Al(III): retrograde endosome-to-Golgi vesicle transport, signal transduction (Pkc1p, cell integrity signaling serine/threonine protein kinase), protein mannosylation (cell wall architecture) (deletome, Kakimoto et al. 2005),
- As(III): shikimate and serine, threonine, glutamate biosynthesis (deletome; Haugen et al. 2004), cytoskeleton, DNA repair, secretory pathway and vacuole function, osmoregulation (deletome; Dilda et al. 2008), cell cycle regulation, spindle body and microtubule formation, mitochondrion biogenesis and function, lipid and fatty acid metabolism, cytoskeleton (deletome; Thorsen et al. 2009),
- Cd(II): DNA repair and replication (*RAD27* and *DNA2*, deletome, Serero et al. 2008), ESCRT and retromer complexes in the carboxypeptidase Y (CPY) Golgi-to-vacuole transport route, DNA repair (deletome, interactome; Ruotolo et al. 2008), ion homeostasis and transport, sugar/carbohydrate metabolism (deletome; Thorsen et al. 2009), ER stress response (transcriptome; Gardarin et al. 2010),

- Co(II): iron regulon (transcriptome; Stadler and Schweyen 2002),
- Cu(II): metallothionein production, iron transport (transcriptome; Gross et al. 2000), transportation into the late endosomes *via* the carboxypeptidase Y Golgi-to-vacuole transport route (CPY, deletome; Jo et al. 2008), tryptophan biosynthetic pathway (deletome; Jo et al. 2008), metallothionein production, lipid, fatty acid metabolism, methionine metabolism (transcriptome; Yasokawa et al. 2008),
- Fe(II): transportation into the vacuoles through the alkaline phosphatase Golgi-to-vacuole transport route (ALP, deletome; Jo et al. 2008),
- Ni(II): transportation into the vacuoles through the alkaline phosphatase Golgi-to-vacuole transport route, nucleocytoplasmic transport (ALP, deletome, interactome; Ruotolo et al. 2008),
- Se(III): iron deprivation, oxidative stress and protein degradation responses (transcriptome; Salin et al. 2008),
- Zn(II): low-oxygen response, iron metabolism (transcriptome; Lyons et al. 2004), vacuolar assembling and biogenesis, chaperones for protein folding and targeting, some components of the iron regulon (deletome; Pagani et al. 2007).

Deprivation of essential metals like Zn and Fe, may also lead to the reorganization of the metabolic networks in yeasts, and these processes are well-characterized by transcriptome analyses (Lyons et al. 2000, 2004; Shakoury-Elizeh et al. 2004, 2010; Dainty et al. 2008; Wu et al. 2008). Importantly, iron-deficiency stimulates riboflavin overproduction in *Pichia guilliermondii*, which may possess biotechnological significance (Boretsky et al. 2007; Pynyaha et al. 2010).

Cd(II)-stress related “-omics” studies performed with *S. pombe* (Chen et al. 2003; Bae and Chen 2004; Kennedy et al. 2008), *C. albicans* (Enjalbert et al. 2006), *Blastocladiella emersonii* (Georg and Gomes 2007), *Ganoderma lucidum* (Chuang et al. 2009) and *Cadophora finlandica* (Gorfer et al. 2009) also provided a wealth of information on the organization and regulation of fungal metal stress defense systems. Cd(II) stress responses of different fungi share many common features. Nonetheless, the same studies revealed some species-specific responses as well, *e.g.* deletome analysis of *S. pombe* by Kennedy et al. (2008) pointed to the important role of ubiquinone (coenzyme Q10) biosynthesis in the Cd(II)-tolerance. In addition, fission yeast but not budding yeast harbors *CNT5*, encoding a member of the centaurin ADP ribosylation factor GTPase activating protein family, which may contribute to As(III)/Cd(II) tolerance through the maintenance of membrane integrity and the modulation of membrane trafficking (Vashisht et al. 2009), and a Cd(II)-regulated centaurin-type protein was also identified in the genome of *C. finlandica* (Gorfer et al. 2009). In the aquatic fungi *B. emersonii* (Georg and Gomes 2007), transcriptome analysis revealed six glutathione S-transferases and six metacaspases, which were induced by Cd(II) exposures. In the ectomycorrhizal basidiomycete *Suillus luteus*, the production of heat shock proteins, a metal transporter, a hydrophobin and proteins with function in ubiquitin-dependent proteolysis were related to Zn(II) detoxification and tolerance (Muller et al. 2007). Mycorrhizal symbioses with *Suillus* spp. is considered now as a suitable tool to cultivate pine trees on Cd(II), Cu(II) and Zn(II) contaminated soils (Adriaensen et al. 2005, 2006; Krzmaric et al. 2009).

It is worth noting that *Aspergillus* spp. possess a robust and delicately regulated stress defense system (Pócsi et al. 2005; Miskei et al. 2009), and data on the molecular background of the high heavy metal tolerance of this genus are also accumulating (Fraser et al. 2002; Cánovas et al. 2003, 2004, 2007; Bučková et al. 2005; Odon et al. 2007; Todorova et al. 2008; Mukherjee et al. 2010). Considering the remarkably wide array of molecular genetic tools available for the genetic engineering of this genus it is reasonable to assume that genetically modified *Aspergillus* strains will appear in the future in versatile metal removal technologies. It is remarkable that the number of publications on *Aspergillus*-based water and soil remediation technologies is already rocketing up owing to the abundance of biotechnological experience with this group of fungi (e.g. Price et al. 2001; Rajendran et al. 2002; Katsifas et al. 2004; Mulligan et al. 2004; Deepa et al. 2006; Sandana Mala et al. 2006; Srivastava and Thakur 2006; Fukuda et al. 2008; Coreño-Alonso et al. 2009; Maheswari and Murugesan 2009; Ren et al. 2009; Sharma et al. 2009; Sun et al. 2010; Taştan et al. 2010). In addition, further spectacular technological progress is clearly foreseeable, e.g. in aspergilli-based nanocrystal producing technologies (Bhainsa and D'Souza 2006; Binupriya et al. 2010; Verma et al. 2010).

Fungi can undergo metal/metalloid induced/controlled apoptosis (Borghouts et al. 2001; Georg and Gomes 2007; Liang and Zhou 2007; Nargund et al. 2008; Azevedo et al. 2009; Chatterjee and Luo 2010). Exposures to toxic metals change the morphology and physiology of mitochondria (Yang and Pon 2003; Liang and Zhou 2007) and, as a consequence, may lead to the initialization of cell death programs (Eisenberg et al. 2007). It is worth mentioning that mitochondria, besides many important cellular functions, play a pivotal role in the control of the iron homeostasis of the cells (Foury and Talibi 2001). Mitochondrial and cellular integrity (and life-span!) can be engineered now by the construction of longevity mutants as demonstrated in *Podospora anserina*, e.g. by the overexpression of *O*-methyltransferase, carotenoid biosynthesis enzymes, misfolded-protein-degrading mitochondrial protease, or by the deletion of the “dynamin-related protein 1”, which regulates mitochondrial division (Scheckhuber et al. 2009). It remains to be seen whether or not such mutations would give the fungal cells an increased resistance against the metal/metalloid-initiated apoptosis as well. Importantly, deletion of *PaMTH1* encoding *O*-methyltransferase resulted in a decreased Cu(II)-tolerance (Kunstmann and Osiewacz 2009).

Finally it remains to be mentioned that “-omics” data are also accumulating on both the plant and the fungus sides of plant-mycorrhizal associations under toxic metal exposures (Ouziad et al. 2005; Aloui et al. 2009), leading to a deeper understanding of the combined plant-fungus metal/metalloid stress responses (Schützendübel and Polle 2002). Undoubtedly, this is the prerequisite of the development of new end effective biotechnologies to combat the increasing toxic metal load of our environment.

Acknowledgement The author is indebted to Dr. M. J. Tamás, University of Gothenburg, for reading critically the manuscript.

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<http://www.springer.com/978-94-007-0427-5>

Cellular Effects of Heavy Metals

Banfalvi, G. (Ed.)

2011, XIV, 348 p., Hardcover

ISBN: 978-94-007-0427-5