Chapter 7
Molecular Mechanisms of Adaptations to High Salt Concentration in the Extremely Halotolerant Black Yeast *Hortaea werneckii*

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7.1 Introduction

As crystalline salt (NaCl) is generally considered hostile to most forms of life, it has been used for centuries as a food preservative. However, halophilic and halotolerant microorganisms can contaminate food that is preserved with salt, and they also inhabit natural hypersaline environments around the world, such as salt lakes and solar salterns. These microorganisms can adapt to extreme concentrations of NaCl, and often to high concentrations of other ions as well (Samson et al. 2004). The great majority of studies on halophilic and halotolerant microorganisms have been dedicated to halophilic Bacteria and Archaea (Brock 1979; Ramos-Cormenzana 1991; Ventosa et al. 1998), and to only one eukaryotic species, the alga *Dunaliella salina* (Oren 2005), as it has been considered that other eukaryotic organisms cannot adapt to these extreme conditions.

These considerations have been based on the fact that halophilic and halotolerant fungi were – until a decade ago – considered extremely rare. They were known only to food microbiologists, who occasionally isolated them from food preserved with high concentrations of salt or sugar, using highly selective media. Since the few known halotolerant fungal species did not show any particular preference for the chemical nature of the solute that was lowering the water potential of the medium used for their isolation (Andrews and Pitt 1987; Hocking 1993; Pitt and Hocking 1997), they were considered xerophiles. The decisive criterion for defining their

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xerophilic properties was the ability to grow on a medium which included 17% NaCl or 50% glucose, corresponding to an a_w of ≤ 0.85 (Northolt et al. 1995).

This conviction remained unchallenged until 2000, when melanized yeast-like fungi were first reported as active inhabitants of brine in solar salterns (Gunde-Cimerman et al. 2000). Later studies revealed that hypersaline environments around the globe also harbor related filamentous melanized fungi and many new or rare fungal species with previously unknown natural ecology (Zalar et al. 1999, 2005a, b, 2007, 2008a, b; Butinar et al. 2005a, b). A decade after the first discoveries of fungi in hypersaline waters, we know that the "hypersaline mycobiota" is represented by phylogenetically unrelated groups of fungi, which appear in different geographic locations at salinities higher than 17% NaCl with relatively consistency. Our understanding of the complex microbial processes in natural hypersaline environments was enhanced by including fungi as additional saprotrophic members of the hypersaline communities.

The description of halophilic and halotolerant fungi in naturally hypersaline environments has provided more appropriate and diverse model organisms for the study of eukaryotic adaptations to hypersaline conditions than the previously used salt-sensitive species such as *Saccharomyces cerevisiae*. We now know that fungi which inhabit natural hypersaline environments have a halophilic behavior that is different from that of the majority of halophilic prokaryotes: with few exceptions, they do not require salt for viability, as they can grow and adjust to the whole salinity range, from freshwater to almost saturated NaCl solutions (Plemenitaš et al. 2008). The term "extremely halotolerant" best describes this versatile type of ecological response, as in the most investigated fungal model organism, the extremely halotolerant black yeast *Hortaea werneckii*, described in this chapter.

7.2 Black Yeasts

Black yeasts or melanized yeast-like fungi or meristematic ascomycetes (Sterflinger et al. 1999) belong to orders *Capnodiales*, *Dothideales*, and *Chaetothuriales* (Crous et al. 2009; Schoch et al. 2009). These orders contain a large number of extremotolerant species that can tolerate extreme temperatures (Wollenzien et al. 1995; Sterflinger 1998; Gunde-Cimerman et al. 2003; Ruibal et al. 2009), high salt concentrations (Gunde-Cimerman et al. 2000; Butinar et al. 2005b), desiccation (Gorbushina et al. 2008; Gueidan et al. 2008), variations in pH and nutrient deficiency (Selbmann et al. 2005), and UV and ionizing radiation (Dadachova et al. 2007). The term "polyextremotolerant" can be used in many instances to describe the remarkable ability of these fungi to colonize a wide variety of different environments and endure a broad range of conditions for multiple ecological parameters, which enable them to colonize even domestic household appliances (Gostinčar et al. 2010, 2011).

This interesting group of organisms has been known since the end of the nineteenth century (de Hoog et al. 1999), although difficulties in their morphological identification together with their slow growth and low competitive ability frequently hindered their isolation and identification.

Although their unusual stress-tolerance is conferred by various specific mechanisms or pre-adaptations, some general traits characteristic for the entire group can be determined. The main characteristic is their black color, which is the result of thick and heavily melanized cell walls. Melanin shields them against adverse conditions and environmental stresses of various kinds; it contributes to virulence in pathogenic species and is a scavenger of reactive oxygen species (van Baarlen et al. 2007). Another important characteristic is the ability of black yeasts to grow meristematically at extreme environmental conditions and form microcolonies (Kogej et al. 2007), in which recolonization of old cells by new ones can occur, resulting in multilayered cell walls and additional protection of newly formed cells (Gorbushina et al. 2003). At less extreme conditions they can often shift between filamentous and yeast-like growth (Slepecky and Starmer 2009), enabling colonization of water and solid substrates. They also often produce general stress-protecting extracellular polysaccharides (Selbmann et al. 2005), which enable formation of biofilms and adherence to biotic and living surfaces. In nutrient limiting conditions, cells can be released from the biofilm, spread and colonize new habitats (van Baarlen et al. 2007).

This particular black yeast ecotype enabled successful colonization of hypersaline environments. The dominant group of fungi in hypersaline waters of the salterns are black yeasts from the ordo Capnodiales (Crous et al. 2009). In hypersaline waters at salinities above 17% NaCl, *H. werneckii* is the dominant species (Gunde-Cimerman et al. 2000).

7.3 *Hortaea werneckii*

The morphology of *H. werneckii* is typical for extremophilic species within the *Capnodiales* (Crous et al. 2009). *H. werneckii* is heavily melanized, has a thick cell wall, and is characteristically polymorphic (de Hoog 1993; Wollenzien et al. 1995; Sterflinger et al. 1999; Zalar et al. 1999). At the highest salinities it exhibits an isodiametric type of thallus expansion, which results in highly resistant, meristematic cell clumps with endogenous conidiation (Sterflinger 1998). At lower salinities it grows as yeast cells or as hyphae, depending on the substrate (Plemenitaš et al. 2008). The main morphological characteristics are presented in Fig. 7.1. Thanks to these adaptive abilities it can grow, albeit extremely slowly, in nearly saturated salt solutions, as well as completely without salt, with a broad growth optimum from 6 to 14% NaCl (Gunde-Cimerman et al. 2000; Plemenitaš et al. 2008).

Due to this polymorphic morphology, *H. werneckii* has received many designations in the past (Plemenitaš and Gunde-Cimerman 2005). Nowadays, its identification is additionally based on molecular characteristics, particularly sequencing of the ITS rDNA region and RFLP markers from SSU rDNA and ITS rDNA regions (de Hoog et al. 1999).

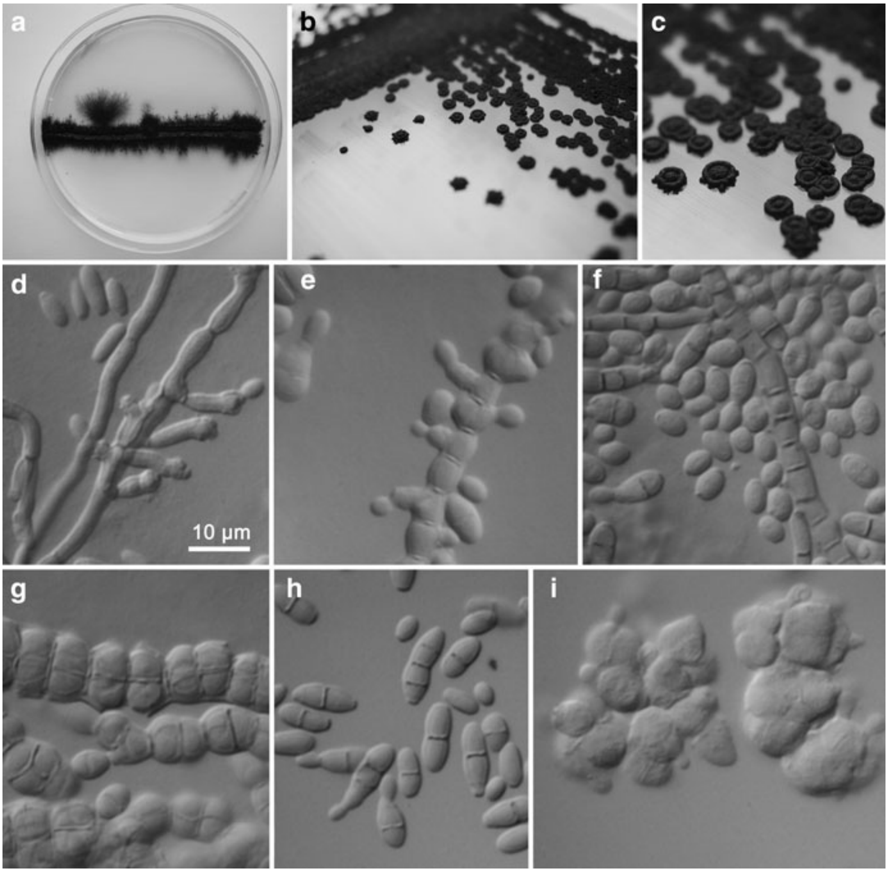

Fig. 7.1 *Hortaea werneckii* in culture: (**a**) Pure culture on MEA with 10% NaCl; (**b**, **c**) Colonies on YNB; (**d**–**f**) Proliferating hyphae on MEA with 5%, 10% and 20% NaCl, respectively; (**g**) Meristematic hyphae on MEA with 20% NaCl; (**e**). Yeast cells on MEA with 5% NaCl; (**i**) Meristematic clumps on MEA with 25% NaCl. The scalebars marked on picture (**d**) is valid also for pictures (**e**–**i**). The pictures were taken by Cene Gostinčar, Rok Miklavčič and Polona Zalar

H. werneckii is at present the best described eukaryotic halophilic model organism. Before it became evident that it is the dominant species inhabiting hypersaline waters, it attracted scientific interest as the causative agent of human *tinea nigra*. This superficial black colonization of the salty human hand, characteristically limited to the greasy stratum corneum, can be removed by vigorous scrubbing (de Hoog and Guého 1998; Göttlich et al. 1995). Nevertheless, there were many speculations about its natural ecology due to its infection potential. More than 10 years after its first discovery in the Slovenian salterns (Gunde-Cimerman et al. 2000) we know that its ecology reflects its halophilic character. *H. werneckii* can be occasionally found as a spoiling agent of food preserved with high salt, it can be isolated with low frequency from seawater-related environments (Zalar et al. 1999), from wood immersed in hypersaline waters (Zalar et al. 2005b) and from surface layers of tropical microbial mats in salterns (Cantrell et al. 2006). There is new evidence that it even inhabits spider webs in Atacama desert caves, together with a new *Dunaliella* species (Azúa-Bustos et al. 2010). However, its primary natural ecological niche is precrystallization and crystallization ponds in eutrophic salterns around the world (Butinar et al. 2005b). It appears in seasonal peaks, which correlate primarily with high environmental nitrogen and phosphorus values. At environmental salinities above 20% NaCl, *H. werneckii* usually represents between 85 and 90% of all isolated fungi, while it can be detected only occasionally when NaCl concentrations are below 10% (Gunde-Cimerman et al. 2000; Butinar et al. 2005b).

7.4 Physiological Responses in *H. werneckii*

We have identified intracellular potassium and sodium content, compatible solutes and membrane characteristics as the key physiological responses in extremely salt-tolerant black yeast *H. werneckii*.

H. werneckii maintains low intracellular Na^+ concentration in spite of the extremely high external salinity. Although the Na^+ to K^+ ratio increased with increased NaCl concentration in the medium, this increase did not follow the increase in external Na^+ concentration (Kogej et al. 2005). These data suggested that *H. werneckii* is well adapted to changes in external ion concentrations, either due to its ability to effectively extrude Na^+ or/and to prevent influx of Na^+.

Second, we found that glycerol is the main compatible solute (Petrovič et al. 2002; Kogej et al. 2005) In *H. werneckii*, intracellular glycerol concentrations correlated well with increases in salinity up to 1.5 M NaCl, whereas at higher salinities intracellular glycerol increased only slightly. We hypothesized that this is due to the rearrangements of the melanin granules on the outer parts of the cell wall of *H. werneckii*, which at lower salinities form a distinct layer, thereby reducing the permeability of cell wall to glycerol. At higher salinities on the other hand, melanization is reduced, resulting in the escape of glycerol from the cell (Kogej et al. 2007). These data suggested the presence of other compatible solutes. Besides glycerol, other low-molecular-weight organic compounds such as polyols erythritol, arabitol, and mannitol, and amino acid derivatives mycosporines were identified as compatible solutes in *H. werneckii* (Kogej et al. 2007). It should be stressed that amino acid derivatives, mycosporines, which accumulate steeply up to 1.0 M NaCl and decrease at higher salinity, have not been previously referred as compatible solutes in fungi (Oren and Gunde-Cimerman 2007).

Salt-induced changes in cellular membranes in *H. werneckii* differ considerably from responses previously observed in the cellular membranes of salt-sensitive *S. cerevisiae* (Turk et al. 2004, 2007). While the sterol-to-phospholipid ratio increases in *S. cerevisiae* with increased salt concentration, in *H. werneckii* this ratio did not alter much. On the other hand, salt stress induced an increase in fatty acid unsaturation and length. Increased salinity was accompanied by a decrease in C16:0 together with an increase in *cis*-C18:$2^{\Delta 9,12}$ (Turk et al. 2004). Such adaptations were earlier proposed to raise membrane fluidity at high salt concentrations (Russell et al. 1995). Indeed, in *H. werneckii*, changes in the fatty acid composition resulted in high plasma membrane fluidity over a wide range of NaCl concentrations. These results indicated high intrinsic salt stress tolerance, and were in good agreement with ecophysiological data and the dominance of *H. werneckii* in hypersaline waters of salterns.

7.5 Molecular Adaptations in *H. werneckii*

7.5.1 Differential Gene Expression

Fluctuation in external salinity forces eukaryotic cells to counteract the osmotic pressure by protective biochemical processes of their protein products, which is first reflected in changes in gene expression. In accordance with physiological responses to increased NaCl concentrations in *H. werneckii,* expression of genes involved in these responses at the level of membranes, ion homeostasis and glycerol synthesis were studied.

An overall increase in fatty acid chain length and unsaturation at increased salinity was observed in *H. werneckii,* with a decrease in C16:0 fatty acids and an increase in *cis*-C18:$2^{\Delta 9,12}$ fatty acids. In accordance with these results was the identification of genes encoding Δ^9-, Δ^{12}-desaturases, and elongases. We found that the genome of *H. werneckii* contains two copies of elongase *HwELO1* and two copies of desaturase *HwODE12*. The expression of these genes was upregulated with an increase in NaCl concentration, with the exception of one isoform of elongase (*HwELO1B*) (Gostinčar et al. 2009).

In line with the assumption that *H. werneckii* maintains low Na^+ due to effective Na^+ exclusion, we have identified an ENA-like ATPases. As revealed by the phylogenetic analysis, the HwENA proteins belong to the group of fungal P-type ATPases, which are phylogenetically older than the Na^+/K^+ ATPases of the salt-sensitive *S. cerevisiae*, and of the salt-tolerant *Debaryomyces hansenii*. The genome of *H. werneckii* contains two genes, *HwENA1* and *HwENA2*, encoding ENA-like ATPases. The expression of both identified genes is responsive to increased salt concentration and to increased pH, both characteristic of the natural environment of *H. werneckii* (Gorjan and Plemenitaš 2006). These results suggested that HwENA ATPases might be involved in the mechanism of the adaptation of *H. werneckii* to its natural environment.

Accumulation of glycerol in a cell is a combination of endogenous synthesis and glycerol uptake from the medium. Like in *S. cerevisiae*, where increased synthesis of glycerol is regulated by the increased expression of glycerol-phosphate dehydrogenase, which produces glycerol-phosphate from dihydroxyacetone-P, we also found salt-dependent increased expression of the gene coding for a putative glycerol-P dehydrogenase in *H. werneckii*. Extremely salt-tolerant *H. werneckii* codes for two salt-induced *GPD1* genes, with similar gene transcription regulation and with 98% amino-acid sequence identity between these paralogs. Both genes are expressed differentially at increased salinity (Lenassi et al. 2011).

Changes in external osmolarity in *S. cerevisiae* as well as in other fungi are sensed by special sensory proteins, which transmit the signal through the HOG signal transduction pathway and regulate the transcription of osmoresponsive genes. In *H. werneckii,* we have identified key components of the HOG pathway: soluble histidine kinase HwHHK7 (Lenassi and Plemenitaš 2007) and transmembrane HwSho1 (Fettich et al. 2011), both putatively involved in sensing high salinity, and three kinases of the MAP kinase module: MAPKKKHwSte11, MAPKK HwPbs2 (data not published), and terminal MAP kinase HwHog1 (Turk and Plemenitaš 2002; Lenassi et al. 2007). Furthermore, we demonstrated that genes like *HwENA1* and *HwGPD1*, both involved in physiological responses to increased salinity in *H. werneckii*, are controlled by activated MAP kinase HwHog1 (Vaupotič and Plemenitaš 2007a).

While searching for novel osmoresponsive genes, we assessed the differential gene expression of extremely halotolerant black yeast *H. werneckii* at the global level. Suppression subtractive hybridization (SSH) is a powerful technique to create a library of differentially expressed genes even from an organism without sequenced genome. We therefore constructed a cDNA subtraction library of *H. werneckii* adapted to hypersaline environment at 3 M and 4.5 M NaCl and identified an uncommon osmoprotective set of 95 differentially expressed genes, presented in Fig. 7.2. Their majority of has not been previously connected to hypersaline adaptation in *S. cerevisiae*. Novel genes putatively connected with extreme osmoadaptability of *H. werneckii* were identified. The analysis of transcriptional responses in hypersaline-adapted and hypersaline-stressed cells revealed that only a few genes responded to acute salt-stress, whereas all were differentially expressed in adapted cells (Vaupotič and Plemenitaš 2007a). It is also of interest that the majority of genes were upregulated at increased NaCl concentrations (Fig. 7.2), while only few, mostly connected to the cell cycle and cell wall biosynthesis, were downregulated (Fig. 7.2). As shown in Fig. 7.2, a high proportion of the differentially expressed genes is involved in metabolism and many of them are connected to the mitochondria. A proteomic study of the mitochondria further revealed preferential accumulation of energy metabolism enzymes in the hypersaline environment (Vaupotič et al. 2008a).

We were particularly interested in genes which are regulated by the MAP kinase HwHog1. By using a chromatin immunoprecipitation (CHIP) assay we demonstrated that more than one third of the differentially expressed genes were associated with the MAP kinase HwHog1. Based on our data on identification and characterization of the components of the HOG signaling pathway in *H. werneckii* and the identified genes that are regulated by the activated HwHog1, we propose the model shown in Fig. 7.3.

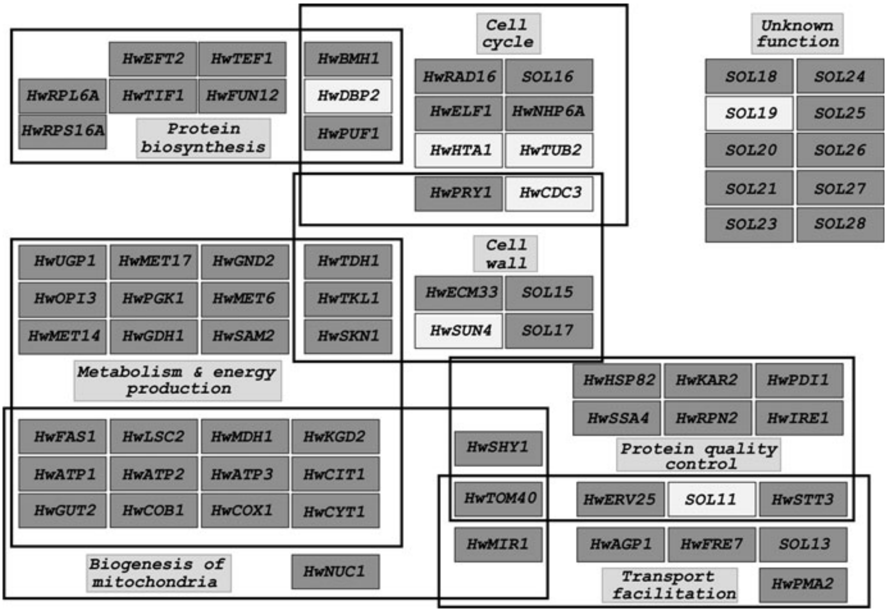

Fig. 7.2 Differentially expressed genes in *H. werneckii* grouped according to their respective functions. *Dark gray squares* represent up-regulated genes, *light gray squares* indicate down-regulated genes

7.5.2 *Proteins as Determinants of Halotolerance in* H. werneckii

At the protein level, we have identified two proteins in *H. werneckii* that are particularly responsive to increased NaCl concentration: hydroxy-methylglutaryl coenzyme A reductase (HwHMG R) and 3′-phosphoadenosine-5′-phosphatase (HwHal2), which is encoded by the *HAL2* gene.

7.5.2.1 HwHMG R

When studying the regulation of hydroxy-methylglutaryl coenzyme A reductase (HMG R), a key regulatory enzyme in the biosynthesis of sterols, we found a specific regulation pattern of this enzyme in *H. werneckii*. The activity and level of HMG R were investigated in *H. werneckii* and in other selected halophilic fungi isolated from solar saltpans. Representative fungi from the orders *Capnodiales Dothideales, Eurotiales*, and *Wallemiales* showed every a similar pattern of HMG R regulation, which differs from the pattern observed in salt-sensitive and moderately salt-tolerant yeasts. In all halophilic fungi studied, the HMG R amounts and activities were the lowest at optimal growth salinity and increased under hyposaline

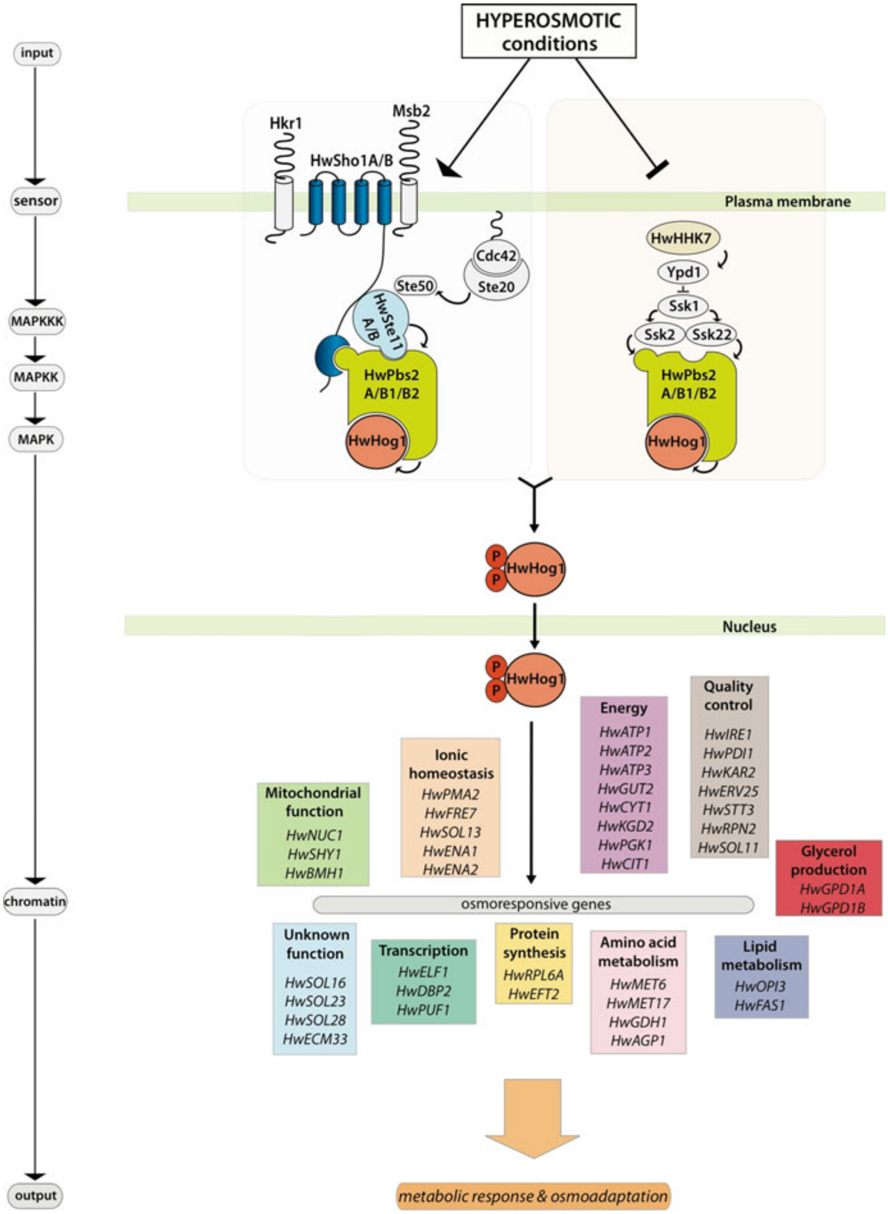

Fig. 7.3 HwHog1 MAP kinase of the HOG signal transduction pathway regulates the transcription of many osmoresponsive genes in *H. werneckii*

and hypersaline conditions. We therefore propose that HMG R could serve as one of the hallmarks in distinguishing between halophilic and non-halophilic fungal species (Vaupotič et al. 2008b).

Our studies also revealed that *H. werneckii* contains two HMGR isoenzymes located in the mitochondria (HwHmg1) and the endoplasmic reticulum (HwHmg2). We demonstrated that the activity of the microsomal isoenzyme, but not of the mitochondrial one, depends on environmental salinity. The activity of the microsomal HwHmg2 was the highest in hypo-saline and extremely hyper-saline environments, whereas it was down-regulated under optimal growth conditions. This was due to intensive ubiquitination and proteasomal degradation of the HwHmg2. The activity of the truncated mitochondrial HwHmg1 was constant in different growth conditions, suggesting an osmoadaptation-directed fate for mevalonate utilization in *H. werneckii*. As shown by the analysis of prenylated proteins, the salt-dependent HwHMG R activity profile mirrors protein prenylation rather than cellular sterol content, which does not change significantly with changing salinity (Vaupotič and Plemenitaš 2007b).

7.5.2.2 HwHal2

The 3-phosphoadenosine-5-phosphatase encoded by the HAL2 gene is a ubiquitous enzyme required for the removal of 3′-phosphoadenosine-5′-phosphate (PAP) produced during sulfur assimilation in eukaryotes. Inhibition of Hal2 by Na^+ or Li^+ results in PAP accumulation, which is toxic for yeasts. Increased amounts of PAP inhibit enzymes like sulphotransferases (Albert et al. 2000), RNA processing enzymes (Dichtl et al. 1997) and nucleoside diphosphate kinase (Schneider et al. 1998).

Two novel HAL2-like genes, HwHAL2A and HwHAL2B, have been cloned from the saltern-inhabiting extremely halotolerant black yeast *H. werneckii* (Vaupotič et al. 2007). Both HwHAL2 isoforms were inducible upon addition of salt. We clearly demonstrated halotolerance of their respective protein products compared to a homologous protein from salt-sensitive yeast. When HwHal2 proteins were expressed in a salt-sensitive strain of *S. cerevisiae*, an increase in halotolerance to up to 1.8 M NaCl was achieved, that has never been observed before with the HAL2 products of other species. HwHal2 proteins have unique structural features, possibly involved in the evolution of salt resistance. Modeling revealed two loops, META and ANA, which have not been previously identified in other Hal2 proteins. Using genetic and biochemical validation, it was demonstrated that the META sequence motif has the most evident effect on the HwHal2B-dependent salt tolerance (Vaupotič et al. 2007). The identification of HwHal2B, which significantly increases halotolerance in yeast, could provide a promising transgene for improving halotolerance in crop improvement strategies.

7.5.3 Gene Duplications as a Global Response in H. werneckii

Gene duplication in general is an already accepted mechanism of adaptation to various stresses. In yeast, for example, most of the duplicated genes code for membrane transporters and proteins involved in stress responses (Kondrashov et al. 2002).

From our studies it appears that gene duplication is also an important mechanism to combat stress due to fluctuations in environmental salinity in *H. werneckii*. Duplications were demonstrated in all genes involved in so far observed physiological responses in *H. werneckii*. Starting from the components of the HOG signal transduction pathway, in which gene duplications are not common in other fungi, we found gene duplications in putative sensory proteins, soluble histidine kinase HwHHK7 and transmembrane HwSho1, as well as in MAPKKK HwSte11 and MAPKK HwPbs2 (our unpublished data). While the expression of the *HwHHK7A* gene, coding for histidine kinase, increased only slightly with increased NaCl concentration, the expression of the *HwHHK7B* gene was highly salt-responsive in salt-adapted *H. werneckii* cells, as well as in stressed cells. Moreover, the expression profile of the *HwHHK7B* gene after exposure of the cells to hypersaline and hyposaline stress indicated the existence of two types of responses: an early response to hyposaline stress and a late response to the hypersaline stress (Lenassi and Plemenitaš 2007). The genome of *H. werneckii* also contains two copies of the *HwSHO1*genes (Fettich et al. 2011). Although the expression level of these genes does not change significantly with increased salt concentration, it is of importance to stress that *H. werneckii* is to our knowledge the first reported fungus with SHO1 gene duplication, as thus far only one copy of this gene (or none) has been found in the genomes of sequenced fungi (Krantz et al. 2006).

Adaptations at the level of membrane fluidity, ion homeostasis, as well as compatible solutes synthesis is also accompanied by duplications of genes coding for proteins involved in these responses in *H. werneckii*. We demonstrated gene duplications in fatty acid elongases and desaturases, involved in salt-dependent changes in fatty acids structure that correlated well with the salt-dependent changes in membrane fluidity (Turk et al. 2004; Gostinčar et al. 2009). It has been suggested earlier that fatty acid modification serves to raise membrane fluidity at high salt concentrations; thus, the duplication of the desaturases may be part of the adaptations of *H. werneckii* to saline environments, compared to the salt-sensitive *S. cerevisiae* which has only one Δ^9-desaturase and is incapable of Δ^{12} desaturation (Gostinčar et al. 2009).

We also identified two gene copies of the *HwENA1* genes that are differentially expressed and are involved with ion homeostasis. While the transcription of *HwENA1* was induced at higher level when the cells were exposed to salt stress, the expression of the *HwENA2* gene was higher in the adapted cells, suggesting their different roles in ion homeostasis. The genome of *H. werneckii* contains two copies of the *HwGPD1* gene, responsible for glycerol production. As glycerol is a key compatible solute in *H. werneckii*, duplication of the *GPD1* gene, which codes for a key regulatory enzyme in glycerol synthesis, might also be an advantage in the adaptation mechanisms of *H. werneckii*.

Gene duplications were also found in genes coding for proteins that could well be defined as markers of halotolerance, HwHal2A and B and HwHmg1 and 2. While expression of both the *HwHAL2* genes is salt-responsive, in case of HMG R, only the microsomal version (HwHmg2) is regulated by NaCl (Vaupotič and Plemenitaš 2007b; Vaupotič et al. 2007).

Given all of the above examples of gene duplications, it is tempting to speculate that gene duplications in *H. werneckii* provide evolutionary benefit for life in environments with fluctuating salt concentrations.

7.6 Conclusions

The discovery of the black yeast *H. werneckii* as the dominant fungal species in hypersaline waters enabled the introduction of a new model organism to study the mechanisms of salt tolerance in eukaryotes. Studies on the extremely halotolerant *H. werneckii* have revealed its superior mechanisms for adapting to an extremely broad range of salinities, from freshwater to almost saturated NaCl concentrations. Comparisons with salt-sensitive or moderately salt-tolerant fungi showed novel, intricate mechanisms to combat fluctuating salinity. We described novel physiological adaptations on the level of cell wall, melanization, membranes, ion homeostasis, and compatible solute strategy. On the molecular level we observed gene duplications, novel genes that are differentially expressed at different NaCl concentrations and regulated by the key MAP kinase of the HOG signaling pathway HwHog1, and we identified proteins as appropriate markers of halotolerance. Studies on *H. werneckii* also revealed a new insight into specialized evolutionary mechanisms of adaptation to high environmental salinity and other extremes in the kingdom of fungi.

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