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Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: a molecular perspective at a glance

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Abstract: Halophilic adaptations have been studied almost exclusively on prokaryotic microorganisms. Discovery of the black yeast *Hortaea 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. Its strategies of cellular osmotic adaptations on the physiological and molecular level revealed novel, intricate mechanisms to combat fluctuating salinity. *H. werneckii is* an extremely halotolerant eukaryotic microorganism and thus a promising source of transgenes for osmotolerance improvement of industrially important yeasts, as well as in crops.

Key words: Compatible solutes, differential gene expression, Hal2, halophile, HOG signaling pathway, *Hortaea werneckii*, hypersaline water, ions, melanin.

INTRODUCTION

Water is of vital importance to all organisms. In an aqueous environment of high salt concentration, loss of internal water is a consequence of osmosis (Yancey 2005). Investigations have shown that most strategies of cellular osmotic adaptations are conserved from bacteria to man (Klipp *et al*. 2005). Salt sensitive *Saccharomyces cerevisiae* is a well-studied model system for studies of osmotic adaptation (Blomberg 2000, Hohmann 2002, Mager & Siderius 2002, Klipp *et al*. 2005). While 0.5 M NaCl represents a concentration that is already toxic for *S. cerevisiae*, the same concentration of NaCl is close to growth optimum of another model organism, halotolerant yeast *Debaryomyces hansenii* (Prista *et al*. 1997). Black yeast *Hortaea werneckii* can grow, albeit extremely slowly, in a nearly saturated salt solution (5.2 M NaCl), and completely without salt, with a broad growth optimum from 1.0 – 3.0 M NaCl (Gunde-Cimerman *et al*. 2000). As few extremely salt tolerant eukaryotic microorganisms are known, black yeast in general and *H. werneckii* in particular represent a group of highly appropriate microorganisms for studying the mechanisms of salt tolerance in eukaryotes (Petrovic *et al*. 2002). Since the first isolation of *H. werneckii* from hypersaline water in 1997, we have studied various aspects of its adaptation to saline environment. It has previously been shown that *H. werneckii* has distinct mechanisms of adaptation to high-salinity environments that were neither observed neither in salt-sensitive nor in moderately salt-tolerant fungi (Plemenitaš & Gunde-Cimerman 2005). The most relevant differences studied to date are in plasma membrane composition and properties (Turk *et al*. 2004, 2007), osmolyte composition and accumulation of ions (Petrovic *et al*. 2002, Kogej *et al*. 2005, 2006), melanisation of cell wall (Kogej *et al*. 2004, 2006), differences in HOG signaling pathway (Turk & Plemenitaš 2002), and differential gene expression (Petrovic *et al*. 2002, Vaupotič & Plemenitaš 2007).

ECOLOGY OF *HORTEA WERNECKII*

Hypersaline environments worldwide are dominated by halophilic prokaryotes (Oren 2002). Nevertheless, some rare representatives of *Eukarya* have also adapted to extreme conditions prevailing in man-made salterns and salt lakes. Besides the brine shrimp *Artemia salina,* the alga *Dunaliella,* and some species of protozoa, a surprising diversity of fungi are well adapted to these extreme conditions (Gunde-Cimerman *et al*. 2005).

The dominant group of fungi in hypersaline waters of the salterns are black yeasts (de Hoog 1977) or meristematic ascomycetes (Sterflinger *et al*. 1999) from the order *Dothideales*. *Hortea werneckii* is the dominant black yeast species in hypersaline waters at salinities above 3.0 M NaCl (Gunde-Cimerman *et al*. 2000). Morphology of *H. werneckii* is characteristically polymorphic (de Hoog *et al*. 1993, Wollenzien *et al*. 1995, Sterflinger *et al*. 1999, Zalar *et al*. 1999), hence it has received many designations in the past (Plemenitaš & Gunde-Cimerman 2005). Its molecular differentiation is based on the sequencing of the ITS rDNA region and RFLP markers from SSU rDNA and ITS rDNA regions (de Hoog *et al*. 1999).

Hortea werneckii was primarily known as the etiological pathogen of human dermatosis called *tinea nigra*, a superficial infection of the human hand, strictly limited to the salty, greasy *stratum corneum* of the skin (de Hoog & Gerrits van den Ende 1992, Göttlich *et al*. 1995). It was also known as a contaminant of salty food (Mok *et al*. 1981, Todaro *et al*. 1983) and other low-water-activity substrates such as arid inorganic and organic surfaces (Wollenzien *et al*. 1995), seawater (Iwatsu & Udagawa 1988) and beach soil (de Hoog & Guého 1998). Two successive yr of investigations of potential mycobiota in evaporite ponds of solar salterns along the Slovenian Adriatic coast revealed that the primary environmental ecological niche of *H. werneckii* is hypersaline water (Gunde-Cimerman *et al*. 2000, Butinar *et al*. 2005). *Hortea werneckii* was found within the

entire environmental salinity range (0.5 – 5.2 M NaCl), with three prominently expressed seasonal peaks, which correlated primarily with high environmental nitrogen values. At 3 – 4.5 M NaCl, at the highest peak in August, *H. werneckii* represented 85 – 90 % of all isolated fungi, whereas it was detected only occasionally when NaCl concentrations were below 1.0 M. Although it was later also identified in hypersaline waters of eight other salterns on three continents (Gunde-Cimerman *et al*. 2000, Butinar *et al*. 2005, Cantrell *et al*. 2006), it has never been isolated from oligotrophic hypersaline waters nor from athalasso-haline waters of salt lakes and only rarely from hypersaline waters with elevated temperatures (Gunde-Cimerman *et al*. 2005). Its complex polymorphic life cycle enables *H. werneckii* to colonise other ecological microniches in the salterns besides brine, such as the surface and interior of wood submerged in brine (Zalar *et al*. 2005), thick bacterial biofilms on the surface of hypersaline waters, the soil in dry evaporite ponds and the saltern microbial mats (Butinar *et al*. 2005, Cantrell *et al*. 2006).

COMPATIBLE SOLUTE STRATEGY IN THE CELLS OF *H. WERNECKII*

Cells living in natural saline systems must maintain lower water potential than their surroundings to survive and proliferate. Osmotic strategy employed by most eukaryotic microorganisms inhabiting hypersaline environments is based on the cytoplasmic accumulation of "compatible solutes" – low-molecular-weight organic compounds (Oren 1999) and on maintaining the intracellular concentrations of sodium ions bellow the toxic level for the cells. Mechanisms of salt tolerance have been studied in salt-sensitive *S. cerevisiae* (Blomberg 2000) and in a few halotolerant fungi such as *Debaryomyces hansenii, Candida versatilis*, *Rhodotorula mucilaginosa* and *Pichia guillermondii* (Andre *et al*. 1988, Almagro *et al*. 2000, Silva-Graca & Lucas 2003, Prista *et al*. 2005, Ramos 1999, 2005). Although in *D. hansenii* osmotic adjustments of the major intracellular cations occurs in response to osmotic stress (Blomberg & Adler 1992, Ramos 2005), data from the other investigated fungi show that the maintenance of positive turgor pressure at high salinity is mainly due to an increased production and accumulation of glycerol as a major compatible solute (Pfyffer *et al*. 1986, Blomberg & Adler 1992).

Initial physiological studies in *H. werneckii* showed that, in contrast to *D. hansenii,* it keeps very low intracellular potassium and sodium levels even when grown in the presence of 4.5 M NaCl. Interestingly, in *H. werneckii* the amounts of K^+ and Na^+ were the lowest in the cells grown at 3.0 M NaCl. At this salinity of the medium *H. werneckii* still grows well, but most probably this salinity represents a turning point, shown in restricted colony size, slower growth rate and characteristic changes of physiological behaviour (Plemenitaš & Gunde-Cimerman 2005, Kogej *et al.* 2007). Our primary studies showed that glycerol is the most important compatible solute in *H. werneckii* (Petrovic *et al*. 2002), although these authors indicates the possible presence of other compatible solute(s). Further studies have indeed revealed that *H. werneckii,* when grown in hypersaline media, also accumulates a mixture of organic compounds besides glycerol, including the polyols such as erythritol, arabitol and mannitol. They varied in amounts both with the salinity of the growth medium and with the growth phase of the fungal culture (Table 1). However, the total amount of polyols correlated well with increasing salinity mostly for the account of glycerol and during all growth phases (Kogej *et al*. 2007).

When the growth-phase dependence of compatible solutes in *H. werneckii* grown at extremely high salt concentrations was followed, it appeared that glycerol accumulated predominantly during the exponential growth phase and diminished steeply during the stationary phase. On the other hand, the amount of erythritol increased gradually during the exponential growth phase and reached its highest level during the stationary phase. The amounts

Table 1. Compatible solutes in *H. werneckii*. Intracellular amounts of polyols and mycosporine-glutaminol-glucoside (myc-gln-glc) in *H. werneckii* grown at various salinities and measured A. in the logarithmic growth phase; B. in the stationary phase (data from Kogej *et al*. 2007). The values are in mmol per g dry weight.

A.						
	without NaCl	**0.86 M NaCl**	**1.71 M NaCl**	**2.91 M NaCl**	**3.42 M NaCl**	**4.28 M NaCl**
Glycerol	0.244	1.259	2.294	2.458	2.823	2.941
Erythrytol	0.026	0.104	0.314	0.309	0.252	0.275
Arabitol	0.315	0.165	0.043	0	0	0
Mannitol	0.249	0.155	0.018	0	0	0
Myc-gln-glc	0.011	0.003	0.008	0.004	0.003	0.003
B.						
	without NaCl	**0.86 M NaCl**	**1.71 M NaCl**	**2.91 M NaCl**	**3.42 M NaCl**	**4.28 M NaCl**
Glycerol	0.021	0.102	0.021	1.243	1.225	0.929
Erythrytol	0.016	0.420	0.597	0.728	0.557	0.544
Arabitol	0.128	0.067	0.004	0	0	0
Mannitol	0.443	0.087	0	0	0	0.37
Myc-gln-glc	0.060	0.159	0.146	0.036	0.024	0.019

of other compatible solutes remained low, thus the total amount of polyols decreased during the stationary phase. In the stationary growth phase, *H. werneckii* also accumulated different amounts of two different mycosporines in addition to polyols. Mycosporines, substances with an aminocyclohexenone unit bound to an amino acid or amino alcohol group, were initially known as morphogenetic factors during fungal sporulation and as UV-protecting compounds (Bandaranayake 1998). The hypothesis that in certain microorganisms the mycosporines or mycosporine-like amino acids might play a role as complementary compatible solutes (Oren & Gunde-Cimerman 2007) was lately confirmed for *H. werneckii* with identification of mycosporine-glutaminol-glucoside in produced during the stationary growth phase. This mycosporine accumulated steeply from up to 1.0 M NaCl, and was decreasing at higher NaCl concentrations (Kogej *et al*. 2006). This pattern corresponded with the growth curve of *H. werneckii*. Given their lower content in the cells (Table 1B), they probably do not have as significant a role in osmoadaptation as polyols, but they still contribute to the internal osmotic potential.

CELL-WALL MELANISATION REDUCES GLYCEROL LOSS IN *H. WERNECKII*

Cell walls of black yeasts are melanised. *Hortea werneckii* synthesises a 1,8-dihydroxynaphthalene-(DHN)-melanin under saline and non-saline growth conditions (Kogej *et al*. 2004, 2006). The ultrastructure of melanised cells was compared to the ones grown in the presence of the melanisation inhibitor tricyclazole (Andersson *et al*. 1996). In melanised *H. werneckii* cells, melanin was observed as electron-dense granules in or on the electron-translucent cell walls, whereas the cells with blocked melanin biosynthesis either had no electron-dense granules or these were smaller and lighter in colour. In cells grown without NaCl, melanin granules were deposited in the outer layer of the cell wall forming a thin layer of melanin with separate larger granules. When grown at optimal salinity, *H. werneckii* formed a dense shield-like layer of melanin granules on the outer side of the cell wall. At higher salinities the melanin granules were larger and scarce, and they did not form a continuous layer. In conclusion, *H. werneckii* is highly melanised at low salinities close to the growth optimum, whereas melanisation is reduced at higher salinities (Kogej *et al*. 2007).

We hypothesised that melanin might have a role in the osmoadaptation of *H. werneckii*. A physiological response of *H. werneckii* to the elevated concentrations of NaCl is hyperaccumulation of glycerol in the cells. Compared to other uncharged polar molecules, glycerol has a high permeability coefficient for passage through the lipid bilayers due to its small molecular mass. Therefore, eukaryotic cells using glycerol as a compatible solute combat this either by accumulation of the lost glycerol by transport systems (Oren 1999), which is energetically costly, or by a special membrane structure (high sterol content or reduced membrane fluidity (Oren 1999). For example, in the halophilic alga *Dunaliella*, the lowered membrane permeability for glycerol is correlated with its high sterol content (Sheffer *et al*. 1986, Oren 1999).

Although in *H. werneckii* the ergosterol as the principal sterol together with 23 other types of sterols (Turk *et al*. 2004) constitute the most distinct lipid fraction of cell membranes (Mejanelle *et al*. 2001), the total sterol content remains mainly unchanged with increased salinity. In addition, the plasma membrane of *H. werneckii* is significantly more fluid over a wide range of salinities in comparison with the membranes of the salt-sensitive and halotolerant fungi (Turk *et al*. 2004, 2007). *Hortea werneckii* can thus grow at very high salinities, which require high intracellular amount of glycerol, but at the same time it maintains a very fluid membrane and constant sterol content. It seems that instead of modifying its membrane structure, *H. werneckii* uses a modification of the cell-wall structure to reduce glycerol leakage from the cells. The cell-wall melanisation namely minimises glycerol loss from the cells: as melanin granules form a continuous layer in the outer part of the cell wall, they create a mechanical permeability barrier for glycerol by reducing the size of pores in the cell wall (Jacobson & Ikeda 2005), and thus improving glycerol retention. At optimal salinities *H. werneckii* probably maintains a balance between energetically cheap production of glycerol, which partially leaks out of the cells and therefore needs to be recovered, and by energetically more costly synthesis of other compatible solutes, which escape less easily from the cells and are therefore retained more efficiently. Melanised cell walls reduce the energy needs of *H. werneckii* by retaining the glycerol in the cells. At higher salinities, where melanisation is diminished, higher energy demands of *H. werneckii* are reflected in reduced growth rates and biomass yield at salinity above 3.0 M NaCl (Kogej, unpubl. data). Perhaps the higher proportion of polymorphic cells observed at the increased salinity is another mechanism for reducing glycerol leakage when melanisation is diminished.

As mentioned above, *H. werneckii* maintains a highly fluid membrane also at increased salinities: it decreases C16:0 and increases *cis*-C18:2$^{\Delta 9,12}$ fatty-acyl residues of the membrane lipids (Turk *et al*. 2004), a phenomenon, which is otherwise observed in cells, subjected to low temperatures. A molecular mechanism contributing to such an adaptation mode is partly enabled by the salinity-regulated expression of genes involved in fatty-acid modification. In *S. cerevisiae*, such a response has been observed for genes encoding a Δ^9-desaturase (*OLE1*) and two long-chain fatty-acid elongases (*ELO2*, *ELO3*) (Causton *et al.* 2001). Recently, multiple copies of genes encoding desaturases and elongases were identified in the genome of *H. werneckii*. Their expression pattern, which was determined at different salinities and osmotic stresses, suggests that desaturases and elongases play an important role particularly after sudden (acute) changes in environmental salinity (Gostinčar, unpubl. data). Gene duplication observed in desaturases, elongases and many other genes in *H. werneckii* (see below) has already been accepted as a general mechanism of adaptation to various stresses also in other organisms. In *S. cerevisiae*, for example, most of the duplicated genes are membrane transporters and genes involved in stress response (Kondrashov *et al*. 2002).
By modifying the cell-wall structure instead of lowering the membrane fluidity, *H. werneckii* can maintain high membrane fluidity even at high salinities, which might be one of the factors enabling its growth at decreased water availability.

SENSING THE INCREASED OSMOLARITY - THE HOG SIGNAL TRANSDUCTION PATHWAY IN *H. WERNECKII*

Multiple signaling pathways allow organisms to respond to different extracellular stimuli and to adjust their cellular machinery to changes in the environment. The sensing of changes in environmental osmolarity is vital for cell survival. In *S. cerevisiae*, the pathway for the sensing of osmolarity changes is known as the high-osmolarity

glycerol (HOG) signaling pathway, and is one of the best understood mitogen-activated protein kinase (MAPK) cascades. Upon osmotic stress, the osmosensors Sho1 and Sln1 stimulate this pathway by two distinct mechanisms, converging the signal at the MAPK kinase Pbs2, which phosphorylates its downstream MAP kinase Hog1, a key MAP kinase of the pathway (Hohmann 2002, O'Rourke *et al*. 2002, Westfall *et al*. 2004). Phosphorylated Hog1 controls the transcription of a family of osmoresponsive genes (Tamas *et al*. 2000, Yale & Bohnert 2001, Proft *et al*. 2006).

Hortea werneckii's ability to adapt to a wide range of salinities indicates the presence of an efficient system that can both sense and respond to these changes. The existence of a signaling pathway similar to the *S. cerevisiae* HOG pathway was demonstrated by identification of putative sensor proteins HwSho1 and histidine kinase-like osmosensor HwHhk7, together with two MAP kinases: MAPKK HwPbs2 and the final MAPK HwHog1 (Lenassi *et al*. 2007, Turk & Plemenitaš 2002). We found that the genome of *H. werneckii* contains one copy of the *S. cerevisiae* homologue gene for the osmosensor Sho1, *HwSHO1.* When compared to other known Sho1 proteins, HwSho1 shows a distinct membrane topology with inverted orientation, suggesting different localisation of HwSho1. To obtain better insight into the role of the HwSho1, the protein was expressed in *S. cerevisiae sho1* mutant strain. We demonstrated that the HwSho1 protein can rescue the osmosensitivity of the *S. cerevisiae sho1* mutant, despite its much lower binding affinity to the scaffold protein Pbs2, when compared to the binding affinity of *S. cerevisiae* Sho1 to Pbs2. It appears that the affinity of binding between HwSho1 and Pbs2 depends not only on the SH3 domain at the C-terminus of HwSho1, but also on the amino-acid sequence surrounding the domain. We also assessed the salt-dependent gene expression and found that the expression of *HwSHO1* is only weakly salt-responsive. We proposed that a preferred role of HwSho1 is in general cellular processes rather than in quick responses to the changes in osmolarity (Lenassi, unpubl. data).

The genome of *H. werneckii* contains two copies of histidine kinase genes with the putative role in osmosensing (Lenassi & Plemenitaš 2007). As many of the *H. werneckii* genes that have so far been associated with adaptation to high osmolarity are present in two copies in the genome (Plemenitaš & Gunde-Cimerman 2005), perhaps the histidine kinase duplication could be beneficial for *H. werneckii* living in environments with fluctuations in salt concentration. A comparison of the translated nucleotide sequence of the product from *H. werneckii* with the protein database revealed a high homology with the histidine kinase ChHhk17 from *Cochliobolus heterostrophus*. ChHhk17 and the related BfHhk17 of *Botryotinia fuckeliana* are members of the group 7 of fungal histidine kinases. The isolated genes from *H. werneckii* were therefore named *HwHHK7A* and *HwHHK7B*. An inspection of the relative positions of all fungal histidine kinase groups on a phylogenetic tree (Catlett *et al*. 2003) shows that histidine kinase Sln1 from *S. cerevisiae* and HK7 group position close together, indicating late separation from a common ancestor. The most obvious difference between the Sln1 and HK7 group, however, is the intracellular localisation of the proteins. While histidine kinases of the Sln1 group are membrane bound, histidine kinases from HK7 group are soluble, cytosolic proteins. Since the secondary structure of some histidine kinases are known, we could predict the secondary structures of the described domains with a high degree of certainty. We confirmed that HwHhk7A and HwHhk7B isoforms have all the regions necessary to function as eukaryotic hybrid-type histidine kinases (Wolanin *et al*. 2002). No transmembrane domain could be predicted in the HwHhk7 proteins from *H. werneckii*, which distinguished them from the *S. cerevisiae* Sln1 protein with two transmembrane domains.

Transcription of *HwHHK7A* gene was not very responsive to the changes in NaCl concentration. In contrast, the expression of *HwHHK7B* gene was highly salt-responsive, with higher levels of expression through the whole range of salinities when compared to *HwHHK7A* gene expression. Salt-dependent expression pattern of *HwHHK7* indicated the existence of two types of responses, an early response to hyposaline and a late response to hypersaline stress (Lenassi & Plemenitaš 2007). Our data suggest that the high induction of *HwHHK7B* gene expression as an early response to hyposaline stress could be the result of the specialised role of this histidine kinase in response to conditions of modest osmolarity, as has already been demonstrated for the Sln1 (O'Rourke & Herskowitz 2004). These results lead us to speculate that the role of isoform HwHhk7B in the adaptation of *H. werneckii* is mostly in sensing and adapting to the sudden changes of salinity, which are very common in this organism's natural habitat.

The role of Sln1 in the HOG pathway is generally well studied and well evidenced (Hohmann 2002). By contrast, none of the HK7 group protein members has a known function. Interestingly, all other fungal species but *H. werneckii*, which code for HK group 7, are known as plant or human pathogens (Furukawa *et al*. 2005, Nemecek *et al*. 2006). The lifestyle of some plant pathogens has similarities with life in a high osmolarity environment, as they must also be able to adapt to fluctuating osmolarity when invading the victim organism (Han & Prade 2002). As controlling the osmotic response on the cellular level is of great importance to the pathogenicity of fungi, other HK7 group members could also have a role in osmosensing, as it was predicted for HwHhk7B in *H. werneckii.* The absence of hybrid histidine kinases from animals makes these proteins prominent antimicrobial targets (Santos & Shiozaki 2001), thus group 7 of HKs could present novel sites for the development of fungal inhibitors.

Both osmosensors, Sho1 and Sln1 proteins in *S. cerevisiae* transmit the signals to the downstream MAP kinase cascade of the HOG signal transduction pathway (Hohmann 2002). In *H. werneckii*, we found homologues of two MAP kinases: HwPbs2 and HwHog1 (Turk & Plemenitaš 2002). In *S. cerevisiae*, Pbs2 functions both as a MAPK kinase and as a scaffold protein, which recruits multiple proteins involved in the activation of the HOG pathway. Upon activation, Pbs2 then phosphorylates the target kinase Hog1 (Hohmann 2002). In *H. werneckii,* we found two gene copies of *HwPBS2* that are transcribed and translated into three different isoforms: HwPbs2A, HwPbs2B1 and HwPbs2B2. The expression of *HwPBS2A* and *HwPBS2B2* isoforms was increased 4-fold in the cells adapted to 4.5 M NaCl, whereas the expression of *HwPBS2B1* was not salt-responsive. As suggested with RNA polymerase II-chromatin immunoprecipitation (RNAPol-ChIP) experiments and promoter analysis, the higher steady-state concentration of *HwPBS2A* transcript in respect to *HwPBS2B2* is the consequence of the activation of *HwPBS2A* gene transcription. The expression profiles of *HwPBS2* genes suggested the putative role of HwPbs2A and HwPbs2B2 in response to quick adaptation to severe hyperosmotic shock, whereas the role of HwPbs2B1 is in response to moderate stress adaptation (Lenassi, unpubl. data). In contrast to *S. cerevisiae*, we showed that HwPbs2 proteins are not only localised to the cytosol, but they also bind to the plasma membrane at higher salinities (Turk & Plemenitaš 2002). The HwPbs2 complemented the defect of the *S. cerevisiae pbs2* mutant strain only weakly. This could be explained by the absence of the appropriate binding partners for the HwPbs2 isoforms in *S. cerevisiae* and may indicate the existence of specialised roles of multiple isoforms in the HOG signaling pathway of *H. werneckii.* This explanation could be supported by our finding that HwPbs2

isoforms have a conserved kinase domain, but a very diverse scaffold binding part.

Moving downstream through the cascade, we have also identified the *S. cerevisiae* homologue of the key MAP kinase in *H. werneckii* - HwHog1 (Turk & Plemenitaš 2002). As in *S. cerevisiae*, the genome of *H. werneckii* contains only one copy of the *HOG1* gene. The *HwHOG1* open reading frame encodes a protein of 359 amino-acid residues with a predicted molecular weight of 46 kDa and with all of the conserved regions that are specific for the MAPKs, such as the common docking (CD) domain at the C-terminal end, a TGY phosphorylation motif at amino-acid residues 171–173, and an Asp in the active site. The 3-dimensional model of the full-length HwHog1 protein revealed an overall structural homology with other known MAPKs (Turk & Plemenitaš 2002, Lenassi *et al*. 2007). Although the HwHog1 protein shows high homology to the *S. cerevisiae* Hog1, important differences in both activation and localisation of the phosphorylated and non-phosphorylated forms of HwHog1 have been observed. An *in vitro* kinase assay demonstrated that in contrast to *S. cerevisiae*, where Hog1 is activated even at very low salt concentrations, HwHog1 is fully active only at extremely high salt concentrations (Turk & Plemenitaš 2002). *HwHOG1* successfully complemented the *S. cerevisiae hog1* phenotype at increased osmolarity, caused by 1.0 M NaCl, 1.0 M KCl, or 1.5 M sorbitol. We demonstrated not only that the cells expressing HwHog1 have restored tolerance to sodium and potassium ions and to sorbitol, but also that the osmotolerance was restored only in the presence of the MAPKK Pbs2 (Lenassi *et al*. 2007).

The HOG pathway has classically been considered as specific to osmotic stress. Recent studies have suggested that Hog1 can also be activated in response to heat shock, cold stress, oxidative stress, and UV injury (Gacto *et al*. 2003, Panadero *et al*. 2006). To test the response of HwHog1 to these alternative stresses, we analysed the growth ability of *S. cerevisiae* wild-type, *hog1* and *pbs2* strains expressing the HwHog1, after exposure to UV, high pH, H_2O_2, and low or high temperatures. We found that the activation of HwHog1 is less efficient in response to UV stress than in wild-type *S. cerevisiae* (Lenassi *et al*. 2007). However, when both yeasts were exposed to UV irradiation, *H. werneckii* was much more resistant to UV than *S. cerevisiae* (Turk, unpublished). As melanin is a well-known UV protectant, we can speculate that it is responsible for high viability in melanised *H. werneckii*, and therefore, we can also conclude that the activation of the HOG signaling pathway might not be involved in the UV stress response in *H. werneckii*. In contrast, the HOG signaling pathway is important for the oxidative stress in *H. werneckii cells*. *S. cerevisiae* cells expressing HwHog1 are much more resistant to H_2O_2 than wild-type cells. Furthermore, this phenotype depends on the presence of the MAPKK Pbs2. The ability of *H. werneckii* to combat oxidative stress has recently been addressed again, using hydrogen peroxide as the reactive oxygen species (ROS)-generating compound. Exposure to H_2O_2 resulted in a decrease in *H. werneckii* viability at extremely high salt concentrations, suggesting that the level of ROS degradation and resistance determine the upper limits of the salt tolerance of *H. werneckii* (Petrovic 2006). HwHog1 also appears to mediate the response to high-temperature, but not low-temperature stresses. Amongst all tested stresses, only the heat-shock response is independent of the Pbs2 protein (Lenassi *et al*. 2007). These data suggest that heat-shock signals that activate HwHog1 are transmitted via a pathway distinct from the classical HOG pathway, in which this MAPK and the scaffold protein Pbs2 have crucial roles. High temperature is stressful for *H. werneckii*, as has been shown by ecological studies. So far only a few strains of *H. werneckii* with optimal growth at 32°C were isolated, while the majority typically prefers lower environmental temperatures (Cantrell *et al*. 2006). Activation of HwHog1 could be of general importance in regulating the transcription of the gene set that is involved in combating high-temperature stress. In contrast, *H. werneckii* seems to be more adapted to lower temperatures and therefore HwHog1 is not activated upon low-temperature exposure. Likewise, the exposure of cells to elevated pH turned out not to be connected to HOG pathway activation (Lenassi *et al*. 2007).

RESPONDING TO INCREASED OSMOLARITY BY DIFFERENTIAL GENE EXPRESSION

When an organism is subjected to extreme environmental conditions for extended periods of time, physiological and metabolic changes lead to adaptive responses and tolerance that depend on the response mechanisms available to the system. Previous studies on *S. cerevisiae* have suggested a critical role of differential protein expression to counteract changes in environmental salinity (Norbeck & Blomberg 1997, Li *et al*. 2003, Liska *et al*. 2004). In contrast to *S. cerevisiae*, *H. werneckii* is well adapted to fluctuations in NaCl concentrations. Differentially expressed genes in *H. werneckii* cells grown at different salinities therefore represent the transcriptional response of the adapted cells rather than their stress response. By applying a suppression subtractive hybridisation (SSH) technique coupled with a mirror orientation selection (MOS) method, we identified a set of 95 osmoresponsive genes as differentially expressed in *H. werneckii* adapted to moderately saline environment of 3 M NaCl or extremely saline environment of 4.5 M NaCl. Among them, more than half were functionally related to general metabolism and energy production. Thirteen unclassified genes with no orthologues in other species, which we called *SOL* genes, represented a specific transcriptional response unique to *H. werneckii* (Vaupotič & Plemenitaš 2007). The transcriptional induction or repression of approximately 500 genes in *S. cerevisiae* that are strongly responsive to salt stress was highly or fully dependent on the MAPK Hog1, indicating that the Hog1-mediated signaling pathway plays a key role in global gene regulation under saline stress conditions (Posas *et al*. 2000, O'Rourke & Herskowitz 2004). We approached the study of a possible interaction of endogenous HwHog1 with the chromatin regions of identified up-regulated genes in optimal salinity- or hypersaline-adapted *H. werneckii* cells by a chromatin immunoprecipitation (ChIP) assay. Lacking the information about promoter regions for the identified differentially-expressed genes in *H. werneckii*, a ChIP-coding region PCR amplification was performed (Vaupotič & Plemenitaš 2007). Recently, it has been shown that the activated Hog1 in *S. cerevisiae* is associated with elongating RNA polymerase II and is therefore recruited to the entire coding region of osmoinducible genes (Proft *et al*. 2006). HwHog1 cross-linked with the coding region of 36 of the differentially expressed genes. For 34 up-regulated genes, the interaction with HwHog1 was stronger in cells adapted to 4.5 M NaCl, whereas for 2 down-regulated genes the HwHog1-ChIP signal was stronger in cells adapted to 3 M NaCl, showing not only the transcriptional induction but also the transcriptional repression by HwHog1 (Vaupotič & Plemenitaš 2007). Genome-wide expression profiling studies using wild-type and *hog1* mutant *S. cerevisiae* cells were performed to comparatively identify genes whose up-regulation of expression was dependent on Hog1 (Yale & Bohnert

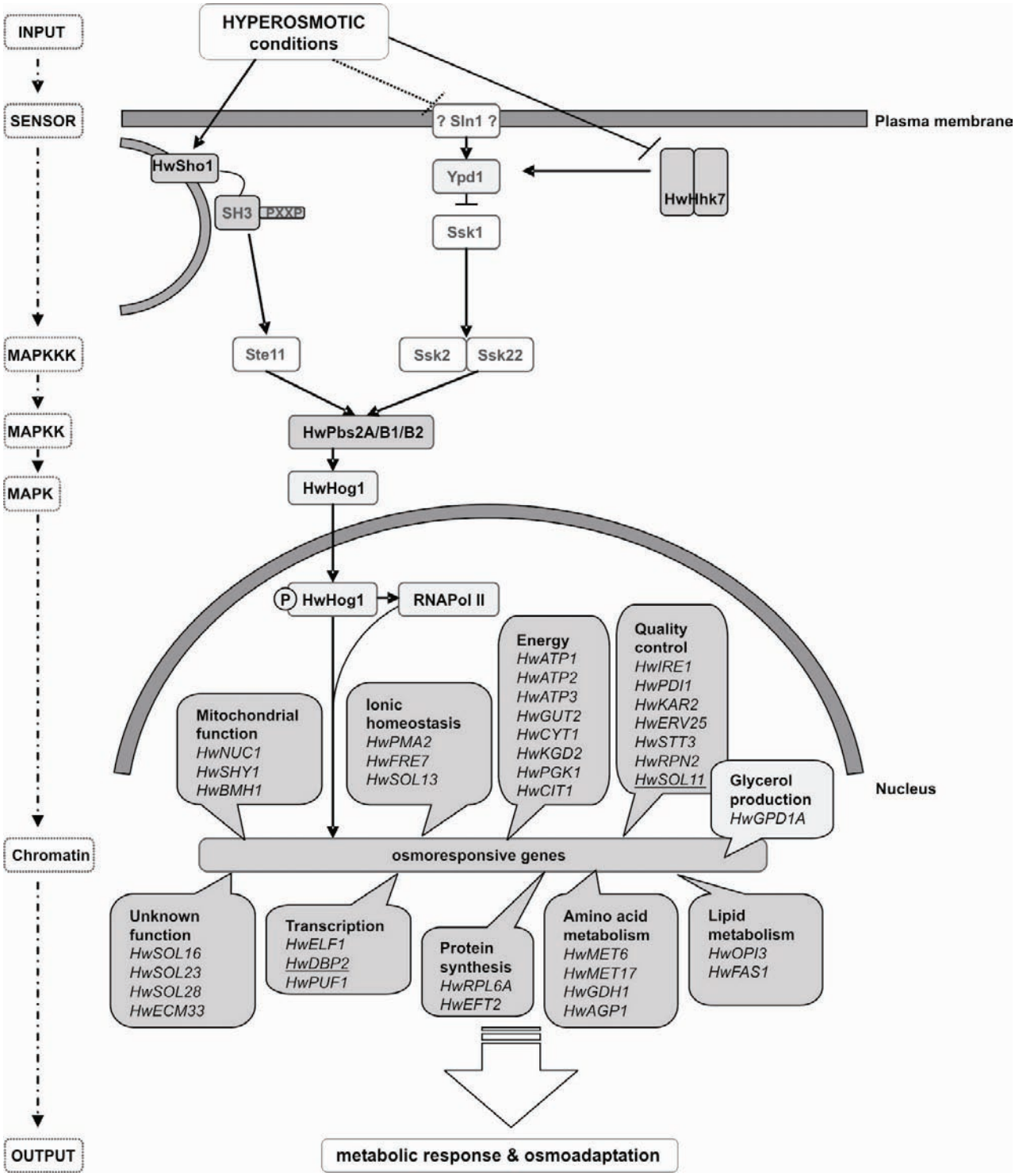

Fig. 1. The model of HOG signaling pathway response during the long-term hypersaline adaptation in the extremely halophilic *H. werneckii*.
Hyperosmotic conditions (4.5 M NaCl) activate the plasma membrane localised osmosensor of the pathway. However, unlike in *S. cerevisiae*, HwSho1 is most likely localised on an inner cell membrane. The Sln1-Ypd1-Ssk1 phosphorelay is much more complex, with an input from at least one more histidine kinase (HwHhk7) and with a questionable role of Sln1 homologue. The signals from both pathways converge at the level of Pbs2 MAPKK homologues (HwPbs2A, HwPbs2B1, and HwPbs2B2). HwPbs2 isoforms putatively activate the HwHog1, a key MAP kinase of the pathway. Upon phosphorylation and translocation into the nucleus, the phosphorylated HwHog1 associates with the chromatin of osmoresponsive genes and thereby promotes (or represses; underlined genes) the transcription, either by recruitment and/or activation of transcriptional factors or by direct association with the RNA polymerase II (RNAPol II), or both. The protein products of HwHog1-interacting osmoresponsive genes belonging to indicated functional groups contribute to the crucial metabolic changes required for successful adaptation to the severe osmotic environment. Although *H. werneckii* has roughly retained the structure of the HOG pathway, it has also developed many distinctive features. The identified components of the *H. werneckii* HOG pathway are shown in dark grey, the evolutionary highly conserved components are shown in light grey, the known components of the *S. cerevisiae* HOG pathway are colorless. HwHog1 responsive genes are: *HwAGP1*, amino acid permease; *HwATP1*, ATPase alpha-subunit; *HwATP2*, ATPase beta-subunit; *HwATP3*, ATPase gamma-subunit; *HwBMH1*, 14-3-3 protein; *HwCIT1*, citrate synthase; *HwCYT1*, cytochrome c1; *HwDBP2*, RNA helicase; *HwECM33*, extracellular matrix protein 33; *HwEFT2*, translation elongation factor 2 (eEF-2); *HwELF1*, transcription elongation factor; *HwERV25*, p24 component of the COPII-coated vesicles; *HwFAS1*, fatty-acid synthase acyl-carrier protein; *HwFRE7*, ferric-chelate reductase 7; *HwGDH1*, glutamate dehydrogenase; *HwGPD1A*, glycerol-3-phosphate dehydrogenase A; *HwGUT2*, FAD-dependent glycerol-3-phosphate dehydrogenase; *HwIRE1*, protein kinase/endoribonuclease; *HwKAR2*, endoplasmic reticulum luminal chaperone; *HwKGD2*, dihydrolipoamide succinyltransferase; *HwMET17*, cystein synthase, *HwMET6*, methionine synthase; *HwNUC1*, mitochondrial nuclease; *HwOPI3*, unsaturated phospholipid methyltransferase; *HwPDI1*, protein disulphide isomerase; *HwPGK1*, 3-phosphoglycerate kinase; *HwPMA2*, plasma membrane proton-exporting ATPase; *HwPUF1*, pumilio-family RNA-binding domain protein; *HwRPL6A*, 60S ribosomal protein 6A; *HwRPN2*, 26S proteasome regulatory subunit; *HwSHY1*, mitochondrial inner membrane protein chaperone; *HwSTT3*, oligosaccharyltransferase catalytic subunit; *SOL11*, mannose-P-dolichol utilization defect 1 protein; *SOL13*, opsin 1; *SOL16*, senescence-associated protein; *SOL23*, hyperosmolarity-induced mRNA 23; *SOL28*, hyperosmolarity-induced mRNA 28.

2001, O'Rourke & Herskowitz 2004, Proft *et al*. 2006). Only the *UGP1* orthologue was also induced in *H. werneckii* cells adapted to 4.5 M NaCl and in cells exposed to a sudden change in salinity. However, in contrast to *S. cerevisiae*, upregulation of *HwUGP1* turned out to be independent of HwHog1 (Vaupotič & Plemenitaš 2007). Other HwHog1-ChIP positive genes in *H. werneckii* were reported for the first time in connection with MAPK Hog1 by our study, reflecting the complexity of HOG signaling pathway. The relative distribution of HwHog1-dependent genes was approximately equivalent among functional categories, except for transcription, cellular transport, signal transduction mechanism, and cell fate functional categories, where the HwHog1-ChIP positive genes represented more than 70 % fraction of tested genes. Only 2 of 10 tested genes with unknown function (*SOL23* and *SOL28*) were HwHog1-ChIP positive.

It has been previously shown that during the HOG response, the nuclear retention and chromatin association of Hog1 in *S. cerevisiae* depends on the co-localisation with general transcription machinery components (Alepuz *et al*. 2001, Alepuz *et al*. 2003). A sequential HwHog1-ChIP analysis (SeqChIP) using primers specific for the genes identified as HwHog1-positive was performed after the primary RNAPol-ChIP in *H. werneckii* (Vaupotič & Plemenitaš 2007). The co-localisation of HwHog1 and RNA polymerase II existed in 17 out of 36 HwHog1-ChiP positive differentially expressed genes. Co-occupation of HwHog1 and RNA polymerase II on target genes resulted in an increased PCR signal in SeqChIP with the accompanying increased level of corresponding transcript in RT-PCR analyses. These observations indicate a stimulating role for HwHog1 and RNA polymerase II co-localisation on the efficiency of transcription of indicated genes in high-salt adapted *H. werneckii* and reflect HwHog1-RNAPolII-chromatin interactions, relevant for the extremely hypersaline conditions, which have so far not been studied in salt-sensitive organisms. Based on our results and in comparison with *S. cerevisiae*, we built the model of HOG signaling pathway in *H. werneckii*, which is shown in Fig.1.

CONCLUSIONS

Black yeast *H. werneckii* is so far the most studied extremely halotolerant eukaryotic model organism. According to our data, *H. werneckii* can be classified as a sodium extruder with an intricate compatible solute strategy, as a response to elevated NaCl concentrations. The main compatible solute of *H. werneckii* is glycerol, which is complemented by erythritol and partially by mycosporine-glutaminol-glucoside in the stationary-phase cells. At low salinities, *H. werneckii* accumulates a mixture of glycerol, erythritol, arabitol and mannitol, whereas glycerol and erythritol prevail at high salinities. At optimal growth salinities, the melanised cell wall helps in retaining high concentrations of glycerol in the cells of *H. werneckii,* despite the highly fluid membrane. The novelty of osmoadaptation of the halophilic fungus *H. werneckii*, probably contributing to its growth at a wide salinity range, is an effective combination of the accumulation of known compatible solutes polyols and of melanised cell walls for improved osmolyte retention.

Our studies confirmed the important role of the HOG signaling pathway in the osmoadaptation and in the stress response of *H. werneckii*. This pathway is activated not only in response to hyperosmotic stress, but also to oxidative and heat stress, both typical for solar salterns. At high salt concentrations, the induction of a completely different set of osmoresponsive genes was observed in *H. werneckii* when compared to salt-sensitive *S. cerevisiae*. Most of these are novel in terms of their interaction with the major transcriptional regulator HwHog1, the mitogen-activated protein kinase of the HOG signaling pathway. Moreover, in *H. werneckii*, HwHog1 mediates not only the early phase of the osmotic induction of many osmo-responsive genes, but it also supports a high RNA-polymerase II-dependent elongation rate of target genes in long-term-adapted cells growing at extremely high salinities. Our studies revealed distinct molecular mechanisms in sensing and responding to changes in environmental osmolarity in *H. werneckii* when compared to the conventional model yeasts, such as salt-sensitive *S. cerevisiae* and moderately halotolerant *D. hansenii*. Differences in protein structure, different intracellular localisation of the components, which are involved in signal transduction, and multiple gene copies, are crucial for these adaptations.

Since salt stress is an increasing threat to agriculture in many productive areas of the world, it is important to bridge the gap between salt toxicity in plants and knowledge of molecular mechanisms of adaptation in extremely halotolerant model eukaryotic cells. Our studies showed that *H. werneckii* is also a promising source of salt tolerant transgenes for agriculture. We identified and characterised two novel isoforms of 3'-phosphoadenosine-5'-phosphatases or Hal2-like proteins from *H. werneckii.* Overexpression of both isoenzymes, HwHal2A and HwHal2B from a low copy number vector in *S. cerevisiae* remarkably increased its halotolerance (Vaupotič *et al*. 2007).

Taken together, an interplaying array of adaptational mechanisms at different levels make *H. werneckii* a very versatile halophile, which is able to grow at a broader salinity range than most known microorganisms. Our findings contribute an important advance in understanding the molecular mechanisms underlying the adaptive response of *H. werneckii*, an increasingly useful model organism for studying the mechanisms of salt tolerance in eukaryotic cells.

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