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Novel 3′-phosphoadenosine-5′-phosphatases from extremely halotolerant *Hortaea werneckii* reveal insight into molecular determinants of salt tolerance of black yeasts

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Abstract

The 3′-phosphoadenosine-5′-phosphatase encoded by *HAL2* gene, is a ubiquitous enzyme required for the removal of the cytotoxic 3′-phosphoadenosine-5′-phosphate produced during sulfur assimilation in eukaryotes. Salt toxicity in yeast and plants results from Hal2 inhibition by sodium or lithium ions. Two novel *HAL2*-like genes, *HwHAL2A* and *HwHAL2B,* have been cloned from saltern-inhabited extremely halotolerant black yeast *Hortaea werneckii.* Expression of both *HwHAL2* isoforms was differentially inducible upon salt. When the *HwHAL2* genes were transferred from such a halotolerant species into the salt sensitive *Saccharomyces cerevisiae,* the resulting organism can tolerate 1.8 M NaCl or 0.8 M LiCl, the highest reported salt concentrations at which *S. cerevisiae* can grow. With genetic and biochemical validation we demonstrated the critical HwHal2B sequence motif—the META sequence—common only to *Dothideales* fungi, with evident effect on the HwHal2B-dependent salt tolerance. These results may have significance for biosaline agriculture in coastal environments.

Keywords: 3′-Phosphoadenosine-5′-phosphatase; Hal2; *Hortaea werneckii*; Halotolerance; Dothideales; Black yeast; Salterns; Extremophiles; Metabolism; 3D structure

1. Introduction

The identification and isolation of several genes in halotolerant species potentially involved in the process of salt tolerance has advanced knowledge of mechanisms involved in adapting to osmotic and ionic stress in yeast. Genetic manipulation of crops to include individual transgenes from halotolerant species and so produce improvement in halotolerance levels could result from such knowledge. The gene products which are modulated by ion influx in high salinity environment are particularly interesting and include the 3′-phosphoadenosine-5′-phosphatase. The enzyme catalyzes the removal of the 3′ phosphate from 3′-phosphoadenosine-5′-phosphate (PAP) or 3′-phosphoadenosine-5′-phosphosulfate (PAPS) forming adenosine-5′-phosphatase or adenosine-5′-phosphosulfate, respectively. It belongs to the enzyme family of phosphomonoesterases, all magnesium-dependent lithium-inhibited phosphatases. In *Saccharomyces cerevisiae*, the gene *HAL2*/*MET22* encodes Hal2, a sodium- and lithium-sensitive PAP phosphatase which is an important determinant of halotolerance in this yeast (Glaser et al., 1993). Hal2 is essential for sulfur assimilation and hence methionine biosynthesis in yeast. When PAPS serves as a sulfate donor, the resulting PAP is subsequently hydrolyzed to adenosine-5′-phosphate and inorganic phosphate by Hal2—a metabolic reaction which is well conserved in bacteria, yeast, plants, and animals (Leustek and Saito, 1999; Murguia et al., 1995; Neuwald et al., 1992; Spiegelberg et al., 1999). During salt stress, the enzyme activity

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of Hal2 is inhibited by lithium ions (50% inhibitory concentration, $IC_{50} = 0.1$ mM) or sodium ions ($IC_{50} = 20$ mM) and PAP accumulates in the cell (Murguia et al., 1995). Elevated concentrations of PAP are very toxic for the cell because it inhibits sulfotransferase (Albert et al., 2000; Murguia et al., 1995), RNA processing enzymes, such as Xrn1 in yeast (Dichtl et al., 1997), and nucleoside diphosphate kinase (Schneider et al., 1998). Therefore Hal2 catalyzes a key metabolic reaction that is limited under high salt stress conditions (Glaser et al., 1993; Serrano, 1996). Overexpression of yeast *HAL2* gene reported to improve salt tolerance in yeast (Glaser et al., 1993) and in plants (Arrillaga et al., 1998). Recently, additional improvements in yeast halotolerance associated with overexpression of the *DHAL2* gene from the halotolerant yeast *Debaryomyces hansenii* (Aggarwal et al., 2005) have been observed.

In our search for better determinants of halotolerance, we have identified a *HAL2* homolog from extremely halotolerant black yeast *Hortaea werneckii* (Ascomycota, Dothideales) first isolated from the hypersaline waters of marine salterns on the Adriatic coast of Slovenia (Gunde-Cimerman et al., 2000). *H. werneckii* was subsequently found elsewhere as a predominant species among a group of halophilic and halotolerant melanized yeast-like fungi, also named black yeasts. This organism can grow actively at salinities up to near-saturated NaCl solution (5.2 M), an ability that is presumed to be based on molecular mechanisms evolved to cope with the extreme variation in ion concentration in the natural habitat of halotolerant fungi.

We have identified and characterized two novel 3′-phosphoadenosine-5′-phosphatase genes, *HwHAL2A* and *HwHAL2B*, in the genome of *H. werneckii*. The expression of the genes was studied at both mRNA and protein levels and increases in halotolerance of the encoded proteins when expressed in *S. cerevisiae* were assessed. Further, based on 3D homology modeling of the HwHal2B structure we have also genetically and biochemically characterized the structural features of the HwHal2B protein which are absent from *S. cerevisiae* Hal2 and appear to be important for halotolerance in other fungi isolated from solar salterns.

2. Materials and methods

2.1. Strains and growth conditions

Cultures of black yeasts *H. werneckii* (MZKI B736), *Aureobasidium pullulans* (EXF 150), *Cladosporium sphaerospermum* (EXF 385), *Eurotium amstelodami* (MZKI A561), *Hortaea acidophila* (CBS 113389), *Phaeotheca triangularis* (MZKI B748), *Trimatostroma salinum* (EXF 295), *Wallemia ichthyophaga* (EXF 994), *Wallemia muriae* (MZKI B952), and *Wallemia sebi* (EXF 757), from culture collections of the Slovenian National Institute of Chemistry (MZKI) or University of Ljubljana, Biotechnical Faculty, Department of Biology (EXF), and the Centraalbureau voor Schimmelcultures Utrecht (CBS), the Netherlands, were used in this study. The reference salt-sensitive strains, *S. cerevisiae* S288C (BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) (Brachmann et al., 1998) and the *S. cerevisiae HAL2/MET22* deletion strain (BY4741: Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOL064c::kanMX4) were both obtained from the Euroscarf Yeast Deletion Strain Collection, Frankfurt, Germany. Cells were grown at 28 °C in a rotary shaker at 180 rpm in a defined medium YNB (0.17% (w/v) Yeast nitrogen base, 0.08% (w/v) Complete supplement mixture (Qbiogene), 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose in deionized water) adjusted to indicated NaCl concentrations and to pH 7.0. Cells were harvested in the mid-exponential phase by centrifugation at 4000*g* for 10 min. For hypersaline stress, *H. werneckii* cells were grown in YNB with 1 M NaCl to OD_{600nm} 1.0 and then the concentration of the medium was adjusted to 3M NaCl. Aliquots of the medium were removed before and 10, 30, 60, 90, and 120 min after the stress. Cells were separated from growth medium by fast filtration through 0.45 μm-pore filter and frozen in liquid nitrogen.

2.2. Amplification of HAL2-like genes from the genome of halotolerant fungi

Highly purified fungal genomic DNA was isolated from mid-exponential phase cells grown in YNB media without salt by the phenol/chloroform/isoamyl alcohol method modified for the DNA isolation from filamentous fungi as described previously (Rozman and Komel, 1994). Partial sequences of *HAL2* orthologs from halotolerant fungi were amplified with touch-down PCR using the degenerate primers 5′-TTCYTIMGIGGIGGICARTAYGC-3′ and 5′-RTGRTCCCAIATYTTYTCCTGGTA-3′, corresponding to conserved amino acid regions in the alignment of known fungal Hal2 orthologs. Fifty nanograms of genomic DNA was used as a template in 50 μL PCRs with Gotaq DNA polymerase (Promega). PCR was performed by 12 touch-down cycles with an annealing temperature from 61 °C to 55 °C followed by 23 cycles with annealing at 55 °C. Expected 500 bp PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced.

2.3. Cloning of the HwHAL2 cDNA and Southern blot analysis

A 302 bp *HwHAL2* PCR product was amplified from *H. werneckii* genomic DNA by PCR using the primers 5′-TTCCTGGTAGTCCTTGCGCA-3′ and 5′-A ATT CGG GGTCCTGTTCTCC-3′. The probe was labeled with [^{32}P]dCTP using the Prime-It random primer labeling kit (Stratagene) according to the manufacturer's instructions and then used as a probe. To clone the *HwHAL2* cDNA, we constructed a *H. werneckii* cDNA library. The total RNA was isolated from mid-exponential cells of *H. werneckii* grown in YNB medium with 3 M NaCl by the classic acid guanidinium thiocyanate/phenol/chloroform method