Identification and characterization of ENA ATPases *HwENA1* and *HwENA2* from the halophilic black yeast *Hortaea werneckii*

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Abstract

Two genes, *HwENA1* and *HwENA2*, which encode ENA-like ATPases in the extremely halotolerant black yeast *Hortaea werneckii,* were cloned and sequenced. Although the expression of both genes is responsive to salt, the transcription of the *HwENA1* gene was induced at a higher level when the cells were exposed to salt stress, and the expression of *HwENA2* gene was higher in the adapted cells, suggesting their different roles in maintaining alkali cation homeostasis. According to the phylogenetic tree based on the amino acid sequences, they represent a new group of fungal *P*-type ATPases. The comparison of both amino acid sequences with other fungal ENA ATPases, together with salt- and pH-responsive gene expression, suggests that newly identified *ENA* genes could be involved in maintaining low Na^+/K^+ content in *H. werneckii*.

Introduction

Hortaea werneckii is a melanized yeast-like fungus, belonging to the *Dothideales, Ascomycota* group. *Hortaea werneckii* can grow in highly changeable saline environments at salinities ranging from 0% to a saturated solution of NaCl [32% NaCl (w/v)] and thus is one of the most halotolerant and adaptable species known among eukaryotes. To date, studies in *H. werneckii* have focused on the changes in morphological features (Zalar *et al*., 1999), membrane properties (Turk *et al*., 2004), compatible solutes (Petrovic *et al*., 2002) and the salt-sensing signal transduction pathway(s) (Turk & Plemenitas, 2002). All these studies indicate the high ability of this fungus to successfully adapt to changing salinities in the environment.

The presence of a HOG signalling transduction pathway (high osmolarity glycerol pathway) which senses and responds to hyper-osmotic shock in the salt sensitive yeast *Saccharomyces cerevisiae* was confirmed in halophilic *H. werneckii*. Homologues of Hog1p, the key kinase of the HOG pathway in *S. cerevisiae*, and an upstream MAPKK Pbs2p were identified (Turk & Plemenitas, 2002). When yeast cells are exposed to high salinity, Hog1p MAP kinase is activated by phosphorylation. Activated Hog1p affects the transcription of many genes, among them ENA genes, which encode K^+/Na^+ *P*-type ATPases (Proft & Serrano, 1999).

Most fungi studied use ENA *P*-type ATPases as one of the mechanisms for K^+ and/or Na^+ export, especially when the cells are exposed to high pH and increased sodium concentration in the environment (Banuelos & Rodriguez-Navarro, 1998; Watanabe *et al*., 1999). All ENA ATPases studied so far have been isolated from organisms which are not adapted to extremely high salinity. In *S. cerevisiae,* four or five ENA ATPases (*Ena1-5*), depending on the strain, mediate sodium efflux processes (Garciadeblas *et al*., 1993), while most other fungi have one or two ENA ATPases. Two ENA ATPases were isolated from *Schwanniomyces occidentalis* (Banuelos & Rodriguez-Navarro, 1998) and halotolerant *Debaryomices hansenii* (Almagro *et al*., 2001), and only one from *Schizosaccharomyces pombe* (Benito *et al*., 2002) and salt tolerant *Zygosaccharomyces rouxi* (Watanabe *et al*., 1999). Recently, a third NcENA ATPase was identified in salt tolerant *Neurospora crassa* (Benito *et al*., 2002). All of these ENA ATPases are plasma membrane proteins. *ENA* genes in *S. cerevisiae* constitute a tandem array of four to five repeats of genes encoding nearly identical proteins. The most important and best-studied component of this system is *ENA1*, which is essential for ion homeostasis and salt tolerance in yeast (Haro *et al*., 1991). While several of the ATPases studied, including ENA ATPases from *S. cerevisiae*, are equally effective in suppressing the sensitivity of mutant cells to K^+ or Na^+, NcEna1p from *N. crassa* is more effective for Na^+.

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When exposed to a hyper-saline environment, many organisms exclude Na^+ ions from the cytoplasm due to the potentially toxic effects (so called excluder organisms), while other, among them halotolerant yeast *D. hansenii*, accumulate relatively high concentrations of Na^+ ions, and these are called includer organisms. Low intracellular contents of sodium and potassium have been detected in *H. werneckii*, even when grown in medium with extremely high NaCl concentration (Kogej *et al.*, 2005), indicating the existence of mechanisms to keep these ions out of the cells. We assumed that *H. werneckii* posses a very efficient export system for Na^+ and K^+. As its natural habitats are alkaline waters of saltern ponds, ENA ATPases most probably play an important role in adaptation to a variety of the salinities in the environment. In this paper we present evidence of the existence of two of ENA-like P-ATPases in *H. werneckii*. The putative function of these genes and the homology to other fungal ENA *P*-type ATPases are discussed.

Materials and methods

Strains and growth conditions

A strain of *H. werneckii* (MZKI B-736) (*Ascomycota, Dothideales*) from the culture collection of the Slovenian National Institute of Chemistry (MZKI) was used in this study. Cells were cultured as described previously (Turk *et al.*, 2001) and harvested in the mid-exponential growth phase ($OD_{600\,nm}$ = 0.8–1.0) by centrifugation at 4000 ***g*** for 10 min. In some experiments, media with different pH values were used. The pH 3.5 was adjusted with tartaric acid, media with pH 7 and 8.5 were supplemented with either 20 mM HEPES or 20 mM TAPS, respectively, and the required pH was adjusted with NaOH.

The salt sensitive *S. cerevisiae* BW31a strain (*ena1-4Δnha1Δ*) (Kinclova-Zimmermannova *et al.*, 2005), was used for heterologous expression of *HwENA1* and *HwENA2* and the *S. cerevisiae* W303.1a (*Mat a his3 leu2 ura3 trp1 ade2*) strain (Almagro *et al.*, 2001) was used as a wild-type control. Yeast cells were grown aerobically in YPD or yeast nitrogene base (YNB) without uracil, supplemented with 2% galactose instead of glucose media at 30 °C.

DNA and RNA isolation

Highly purified genomic DNA was isolated according to the phenol/chlorophorm/isoamyl alcohol method from mid-exponential phase cells grown in YNB media without salt (Rozman & Komel, 1994).

Total RNA was isolated from the adapted cells, cells exposed to salt stress and from the cells grown at different pH using TRI® Reagent RNA Isolation Reagent (Sigma). To study the influence of salt stress, cells grown to mid-exponential growth phase in YNB media with 5% NaCl were then exposed to YNB media with 17% of NaCl for 5–90 min. The effect of pH 3.5 and pH 8.5 was investigated in cells grown in YPD, YPD 5% NaCl and YPD with 17% NaCl to mid-exponential growth phase and then incubated for 2 h in the same media at pH 3.5 or 8.5.

Hortaea werneckii cDNA was obtained by reverse transcription using SuperScript™ RNase H⁻ reverse transcriptase (Invitrogen) according to the manufacturer's recommendations.

Hortaea werneckii genomic library construction

Genomic DNA fragments were generated by partial digestion of *H. werneckii* DNA with *Sau*3AI (Roche). The digestion products were separated on a 1% agarose gel and fragments of 2 kb were recovered (Qiaquick PCR Purification Kit, Qiagen). The genomic *H. werneckii* library was constructed in a pBK-CMV phagemid vector (Zap Ekspress® Predigested Vector Kit), which was packaged using Zap Ekspress® Predigested Gigapack® Cloning Kit, titered and amplified in the *Escherichia coli* XL-blue MRF bacterial strain (Stratagene) according to the manufacturer's recommendations.

Cloning and analysis of the *H. werneckii* ENA P-ATPase genes

An *ENA1*-harbouring DNA fragment (*c.* 750 bp) was obtained by Touch-down PCR amplification. The degenerate oligonucleotides 5′CATCAACGATATCTGCTCCGAAYAARACNGG3′ (priming with phosphorylation motif DKTGTLT) and 5′GGGGTGATCGCCGGTNARCATRTG3′ (priming with ATP-binding motif MLTGD) designed by the CODEHOP program (Rose *et al.*, 1998) were used as primers and *H. werneckii* genomic DNA as the template. The primers were constructed as follows: The protein sequence of *S. cerevisiae* Ena1p from Swiss-Prot database was submitted to the BLAST program on ExPASy/SIB. The fungal Ena protein sequences were selected and the CLUSTALW alignment was performed. The alignment was imported to CODEHOP program. After purification by agarose electrophoresis and Qiaquick PCR Purification Kits (Qiagen), the DNA fragment was labelled with [^{32}P]dCTP using the Prime-It® RmT Random Primer Labelling Kit (Stratagene) according to the manual. *Hortaea werneckii* genomic library screening with a radioactively labelled *ENA1* probe was performed as previously reported (Turk & Plemenitas, 2002). The *H. werneckii* DNA inserts in positive clones were PCR amplified with oligonucleotides T3 and T7. Two genes were obtained, named *HwENA1* and *HwENA2*. The missing 5′ part of the sequence *HwENA2* was obtained by Genome Walker™ Universal Kit (BD Biosciences Clontech). The cDNA sequence of both of the genes was amplified by PCR amplification using *H. werneckii* cDNA as the template. All PCR products were cloned into pGEM®-T Easy Vector (Promega). Sequence analysis of cloned PCR products was

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performed by Sequiserve (Dr W. Metzger, Vatterstetten, Germany). Searches for homologues in DNA and protein sequence databases were performed using the BLAST programs (http://www.ncbi.nlm.nih.gov/blast/), and alignment analysis with the CLUSTALW (http://www.ebi.ac.uk/clustalw/) and CLUSTALX 1.81 (Thompson *et al.*, 1997) program.

Southern blot analysis

A *HwENA1*-harbouring DNA fragment (*c.* 470 bp) was used as a probe. It was obtained by PCR amplification using the oligonucleotides 5′CGCATGCAACATTCTAAGCT3′ and 5′CTTGACCGTGAAGGATTGTC3′as primers and *H. werneckii* genomic DNA as the template. The fragment was ^{32}P-labelled as described above. Twenty-five micrograms of high purity genomic DNA was digested with the restriction enzymes *Eco*RI and *Hin*dIII (Roche) followed by electrophoresis on a 1% agarose gel. The Southern blot transfer was performed according to the manual (Kaufman *et al.*, 1995). Hybridization was carried out as previously reported (Turk & Plemenitas, 2002).

Chromosomal localization of the *HwENA1* and *HwENA2* genes through pulsed-field gel electrophoresis (PFGE)

The isolation of *H. werneckii* chromosomal DNA and PFGE was performed according to Raspor *et al.* (2000). The Southern blot from the PFGE gel was performed according to Bignell & Evans (1996). A hybridization of a chromoblot was performed as described above using radiolabeled probes specific for each of the *HwENA* genes, obtained by PCR amplification.

Real-time PCR (Q-RT)

Real-time PCR was performed using Custom TaqMan gene expression assays (Applied Biosystems 4331348), designed from partial specific sequences of *HwENA1* and *HwENA2* genes, and TaqMan universal PCR Master Mix (Applied Biosystems). Each gene expression assay was a mixture of unlabled PCR primers (HWENA1A-EN1B 5′CGCTGCCTTTGGTGCTT3′ and HWENA1A-EN1BR 5′GCATGCAACATTCTAAGCTTCGT3′ for *HwENA1* gene; HWENA2A-EN2BF 5′TGAAAACACTTCCTCTGGTGCTT3′ and HWENA2A-EN2BR 5′GCGTGCAACATTCTAAGTTTGGT3′ for *HwENA2* gene) and TaqMan® MGB probes (FAM™ dye-labeled: HWENA1A-EN1BM2FAM 5′CTTGGGCTACATCTCG3′; HWENA2A-EN2BM2FAM 5′CTCGGACTGCATCTCG3′). The PCR reaction was performed according to manufacturer's recommendations. TaqMan® Ribosomal RNA Control (Applied Biosystems), which is designed to detect the 18S rRNA gene, was used as an endogenous control. All Q-RT reactions were performed using the Stratagene Mx3000P Instrument.

Plasmid constructions, functional expression of *HwENA1* and *HwENA2* in *S. cerevisiae* and salt tolerance determination

The coding regions of both *HwENA* genes were amplified using the CERTAMP Long amplifications kit (Biotools), mixture containing a combination of proofreading and non-proofreading enzymes (*Tth* and *Pfu* DNA polymerase) to avoid mismatch base pairing during the PCR synthesis; primers containing restriction site for *Eco*RI (5′TTGAATTCAGATGGAAGAAGCTCCCGTGCA3′ as forward primer for both of the genes, 5′GAGAATTCTGTCAAACGAGCCGTAAACCAG3′ as a reverse primer for *HwENA1* gene, and 5′GCGAATTCGGTCAAATGAGCCGTAAACCAA3′ as a reverse primer for *HwENA2* gene) and *H. werneckii* cDNA were used as a template. PCR products were cloned, cut with *Eco*RI enzyme and inserted into expression plasmid pRD53 (GAL1, 10 region (RI-Bam fragment) in SpeI-Bam sites of pRS316 (CEN, URA3) (Plemenitas *et al.*, 1999), cut with the same restriction enzyme. The constructs were cloned in *E. coli* DH5α cells. The orientation of the inserted fragments was tested using T3 or T7 as the first primer and a gene-specific internal primer.

Yeast cells, used for functional expression of *HwENA1* and *HwENA2*, were grown overnight in YPD media at 30 °C and 180 r.p.m. to mid-exponential phase and then transformed with 1 μg of both of the pRD53 constructs and an empty pRD53 as a control, using Alkali – cation yeast transformation kit (Qbiogene) according to manufacturer's protocol. Transformants were selected on YNB plates without uracil (YNB-Ura) and tested with gene specific primers to confirm the presence of the constructs in the yeast cells.

Drop test experiments were performed on solid and liquid YNB-Ura media supplemented with different molar concentrations of NaCl, KCl and LiCl. The effect of pH 5 and 8.5 was also tested. Positive yeast clones were grown overnight in YNB-Ura medium to mid-exponential phase, adjusted to $OD_{600\,nm}$ 0.5, 10-fold serially diluted (1–10^4 dilutions) with fresh medium and spotted in 3 μL onto YNB plates without uracil and supplemented with 2% (w/v) of galactose instead of glucose (YNB-Ura+Gal).

Results and discussion

Although *H. werneckii* can grow in salinities of up to 32% NaCl, it was found that intracellular contents of sodium and potassium are surprisingly low in this microorganism (Kogej *et al.*, 2005). Thus *H. werneckii* must posses a very efficient mechanism to maintain low cation contents inside the cells. Preliminary studies on the isolated plasma membrane have shown that the enzyme activity of the plasma membrane P-ATPases is highly sensitive to salt (Plemenitas & Gunde-Cimerman, 2005; and data not published). Since ENA P-ATPases are involved in ion transport in several

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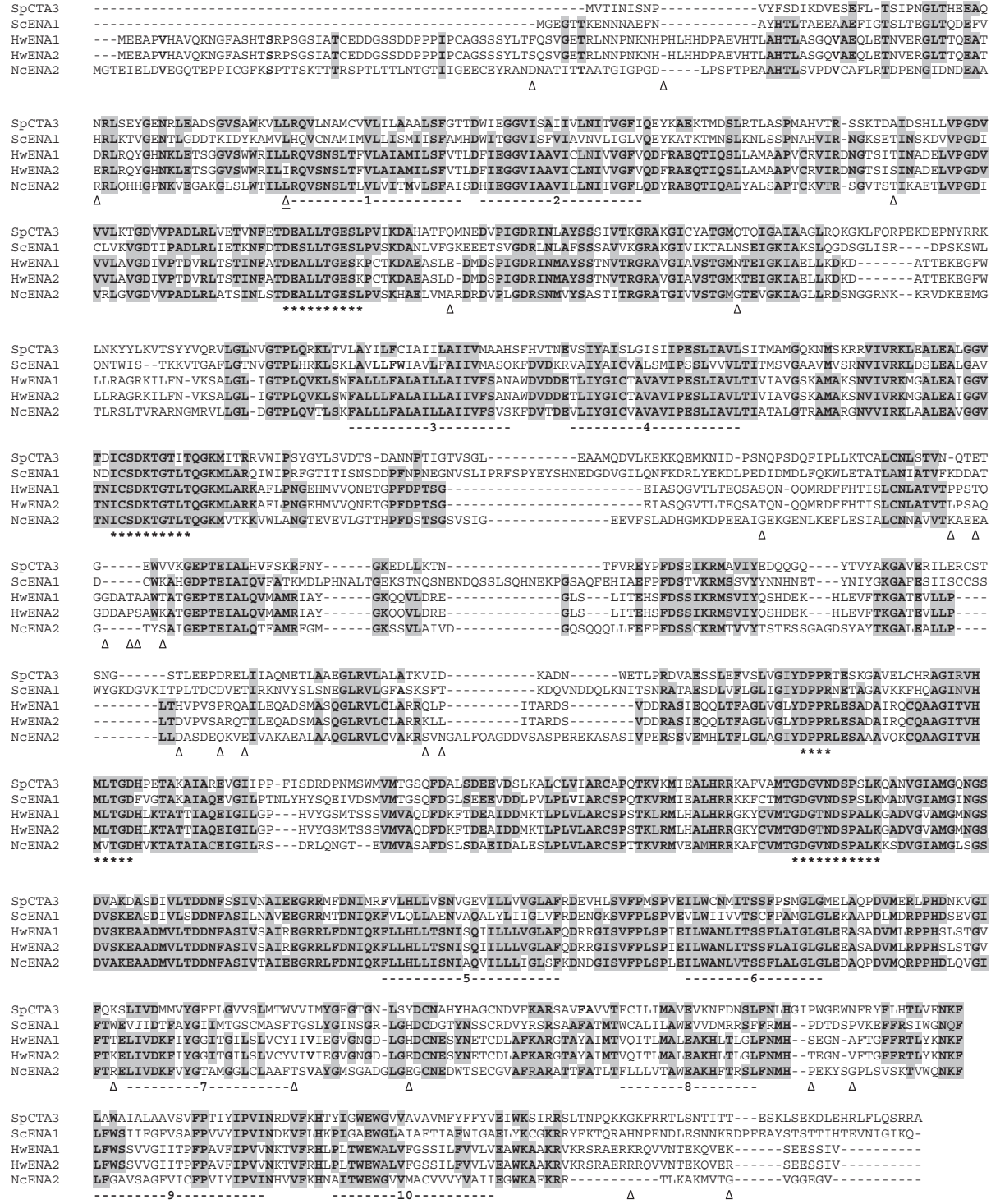

Fig. 1. Deduced amino acid sequences of the coding regions of *HwENA1* and *HwENA2*, aligned with protein sequences of NcEna2p from *Neurospora crassa*, Cta3p from *Schizosaccharomyces pombe* and Ena1p from *Saccharomyces cerevisiae*. Identical amino acid residues are shown by bold characters and highlighted in grey. Ten predicted transmembrane domains are underlined together with the number. Asterisks indicate peptide motifs significant for the functions of Na^+ ATPase; the phosphatase motif (DEXLXTGESL), phosphorylation motif (DKTGTLT), and ATP-binding motifs (DPPR, MLTGD and GDGVNXXPSLK). The differences in amino acid sequences of HwEna1p and HwEna2p are marked with triangles.

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studied fungi, we assumed that ENA-like ATPases exist in *H.werneckii* and play an important role in ion homeostasis during adaptation to changing salinities in the environment.

Isolation and characterization of the *HwENA1* and *HwENA2* genes from *H. werneckii*

Using degenerate primers for the PCR reaction, a 753-bp *HwENA* DNA fragment from *H. werneckii* DNA was amplified. This fragment was then used as a probe for screening the genomic library of *H. werneckii*. Using multiple screening of the library, two nucleotide sequences with ORFs of 3268 bp (GenBank accession number DQ401070) and 3265 bp (GenBank accession number DQ401071) were obtained. It was observed that each of them possessed one exon of 55 bp. The sequences of the isolated genes are very similar, consisting of 92% nucleotide identity. Protein sequences of the HwEna ATPases were deduced from the cDNA sequences of the genes. On the basis of protein sequence homologies of the isolated genes with known fungal ENA ATPases (Fig. 1), the corresponding genes were named *HwENA1* and *HwENA2*, respectively. The amino acid sequences of HwEna1p and HwEna2p are 1071 and 1070 amino acids long and are very similar, with differences in only 26 amino acids (2.4%) (Fig. 1 – marked with triangles).

Southern blot analysis of the genomic DNA was performed with a probe lacking the restriction sites for both of the restriction enzymes used. The bands obtained from genomic DNA cut with *Eco*RI and *Hin*dIII revealed the existence of at least two *HwENA* genes in the genome of *H. werneckii* (Fig. 2a).

Chromoblot analysis was performed to check the localization of *HwENA1* and *HwENA2* in *H. werneckii*. As shown in Fig. 2b, both of the *HwENA* genes are located on the same chromosome. We speculate that they are arranged in tandem, similarly to *S. cerevisiae* where ENA genes constitute a tandem array of four to five genes on one chromosome.

The transcription of *HwENA* genes is salt dependent

Next, the expression of *HwENA1* and *HwENA2* was studied at different NaCl concentrations. Results obtained by Real-time PCR in Fig. 3 show that the expression of *HwENA* genes is responsive to salt. The expression of both *HwENA* genes from the adapted cells of *H. werneckii* was strongly induced in hyper saline environment (25% NaCl), while it was relatively low up to 17% of NaCl) (Fig. 3a). It was found that adapted cells of *H. werneckii* have low sodium and potassium intracellular contents, which do not vary much with increasing extracellular salt concentrations (Kogej *et al*., 2005). We assume that the adapted cells use a variety of molecular mechanisms to keep low intracellular cation contents over a wide range of salinity. Based on our results of

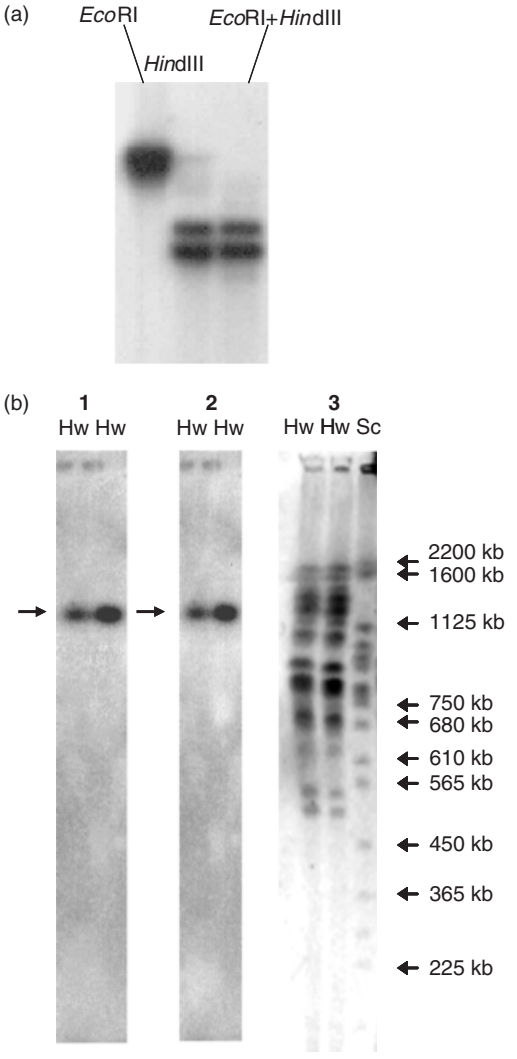

Fig. 2. Determination of the number of *HwENA* gene copies in *Hortaea werneckii*. (a) Southern blot analysis of *H. werneckii* genomic DNA, digested with different restriction endonucleases (*Eco*RI, *Hin*dIII, *Eco*RI+*Hin*dIII). Digested DNA was probed with a radiolabeled *HwENA1* fragment. (b) Chromosomal localization of the *HwENA* genes. (1), (2): Southern blot analysis of *H. werneckii* chromosomes, separated by PFGE. Chromoblot of the gel was probed with radiolabeled *HwENA1* (1) and *HwENA2* (2) fragments. (3): *Hortaea werneckii* chromosomes, separated by PFGE. *Saccharomyces cerevisiae* chromosomes were used as a standard. Hw, *H. werneckii* chromosomes; Sc, *S. cerevisiae* standards.

expression of *HwENA* genes, it seems that HwENA ATPases contribute to these mechanisms only at extremely high NaCl concentrations, especially with the increased expression of *HwENA2* gene (Fig. 3a).

When cells growing at 5% NaCl were exposed to salt stress by a sudden addition of NaCl into the growth medium to 17% NaCl of final concentration, the expression profile differed from the one obtained in the adapted cells. mRNA expression of both genes was induced only after 90 min with

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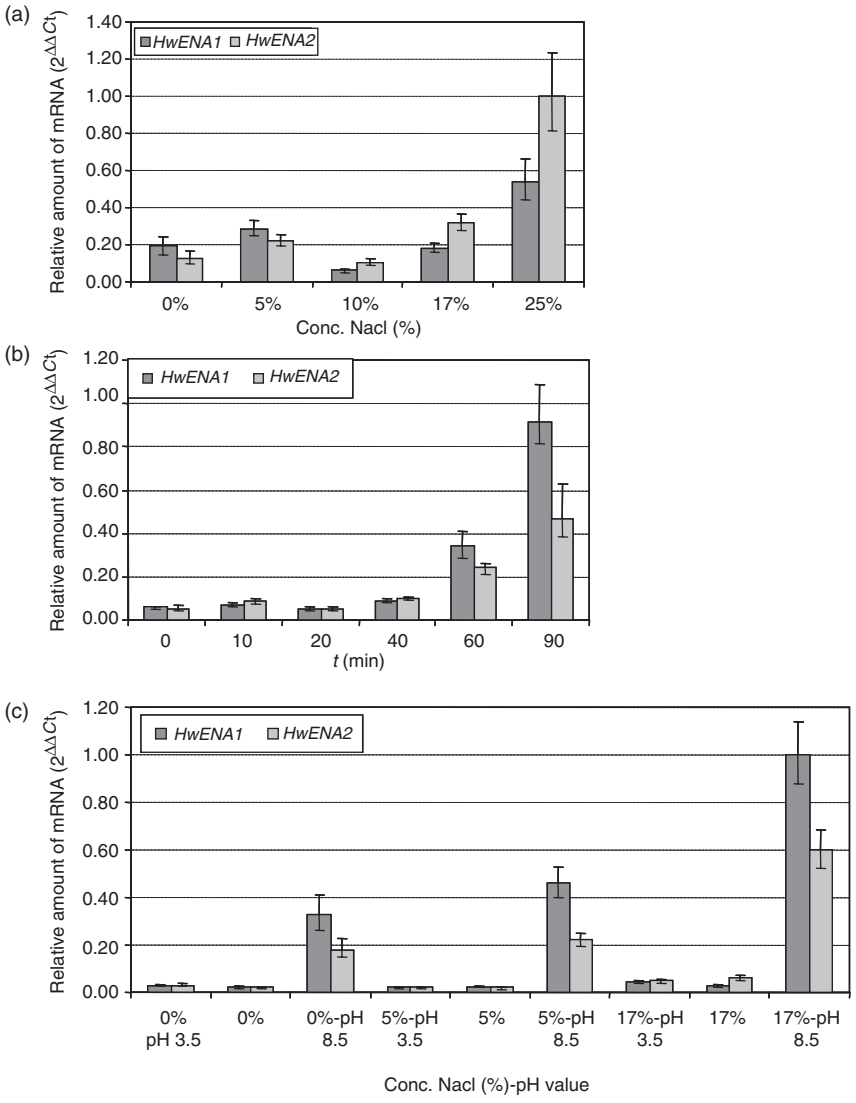

Fig. 3. The expression of *HwENA1* and *HwENA2* genes obtained by real-time PCR. The mean of $2^{\Delta\Delta Ct}$ values are presented, obtained by three real-time PCR experiments in duplicates $\pm$ SEM. (a) The expression of *HwENA1* and *HwENA2* in adapted cells, grown at different concentrations of NaCl in the media. (b) The expression of *HwENA1* and *HwENA2* after salt stress. Cells were grown in YNB supplemented with 5% NaCl and then exposed to sudden increase in salt concentration (17% NaCl final concentration) for 90 min. (c) The expression of *HwENA1* and *HwENA2* at different pH of the media.

the level of *HwENA2* mRNA being lower then *HwENA1* mRNA (Fig. 3b). According to our data obtained in the studies of expression of more then 100 salt-responsive genes in *H. werneckii* (unpublished data), two clearly different responses were observed: while some genes are induced within 10–20 min and are involved in early response, others, like *HwENA* genes, are expressed only after 60–90 min. Therefore, *HwENA* genes are involved in the late/r response of the cells to salt stress, with *HwENA1* being more responsive to sudden changes in the salt concentrations, and *HwENA2* playing more important role in the mechanism of keeping low cation content in adapted cells.

It was found, that pH plays an important role in the function of ENA ATPases (Banuelos & Rodriguez-Navarro, 1998; Benito *et al*., 2000; Almagro *et al*., 2001). To check, if the expression of *HwENA* genes is affected by the pH of the medium, we performed real-time PCR at acidic pH and alkaline pH of the medium. As shown in Fig. 3c, both genes are highly induced in pH 8.5 at increasing NaCl concentrations, while the expression of genes doesn't differ much when exposed to acidic environment. Since the natural habitat of *H. werneckii* are waters of saltern ponds with changing NaCl concentrations and alkaline pH, our results suggest that HwENA ATPases might be involved in the mechanism of the adaptation of this fungus to its natural environment.

HwEna1p and HwEna2p belong to a new group of fungal ENA ATPases

The amino acid sequences of HwEna1p and HwEna2p were compared to other known sequences of fungal ENA proteins from databases (Fig. 1). All five peptide motifs which have been suggested to participate in ENA ATPase functions, the

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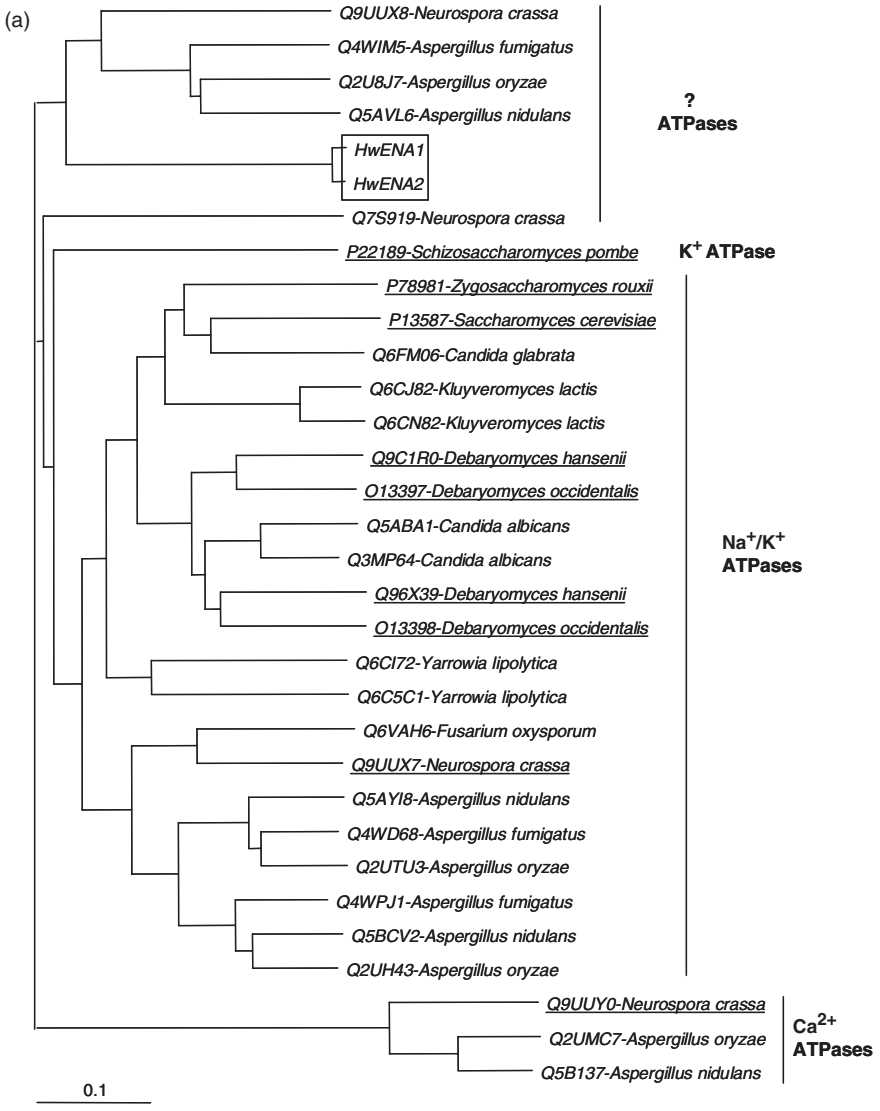

(b)

	M4	M5	M6	M8
NcENA1	SMIPASLVV	ENIAQAGTL	WIIMITSGL	LTWFALFLA
ScENA1	SMIPSSLVV	ENVAQALYL	WIIVVTSCF	MTWCALILA
Afu2g01320	AVIPESLIA	SNIAQVILL	WANLVTSSF	LTFLLLVTA
AO090701000406	AVVPESLIA	gap VVLL	WANLVTSSF	LTFLLLVTA
AN7664.2	AVVPESLIA	gap VILL	WANLVTSSF	LTFLLLVTA
NcENA2	AVIPESLIA	SNIAQVILL	WANLVTSSF	LTFLLLVTA
HwENA1/2	AVIPESLIA	SNISQIILL	WANLITSSF	MTVQITLMA
NCU07966.1	AIIPESLVA	SNVGEVILL	WINMVTSSF	LTWLILLSA
SpCTA3	SIIPESLIA	SNVGEVILL	WCNMITSSF	VTFCILIMA
AO090003000051	AAIPEGLAV	SNIGEVVSI	WVNLVTDGL	TVSLSILVV
AN5743.2	AAIPEGLAV	SNIGEVVSI	WVNLVTDGL	TVSLSILVV
Nca-1 (Nc)	AAIPEGLAV	SNIGEVVSI	WVNLVTDGL	TVSLSILVV

Fig. 4. (a) Phylogenetic tree of putative fungal Na^+ and Ca^{2+} ATPases. The sequences were obtained from UniProt database. The alignment of the sequences was performed using the CLUSTALX 1.81 program (Thompson *et al*., 1997). Accession numbers are listed in Table 1. The function of the underlined ATPases has been confirmed by functional complementation of *Saccharomyces cerevisiae* mutants and cation efflux studies. (b) Sequence alignments of the *P*-type ATPase fragments from *S. cerevisiae* (*ENA1*), *Neurospora crassa* (*NcENA1*, *NcENA2*, NCU07966.1, *nca-1*), *Schizosaccharomyces pombe* (*CTA3*), *Hortaea werneckii* (*HwENA1*, *HwENA2*), *Aspergillus fumigatus* (Afu2g01320), *Aspergillus nidulans* (AN7664.2, AN5743.2) and *Aspergillus oryzae* (AO090701000406, AO090003000051) containing amino acids putatively involved in Ca^{2+} binding in SERCA [a sarco(endo)plasmic reticulum] Ca^{2+} ATPases (highlighted in grey). M4, M5, M6, M8 denote transmembrane fragments.

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Table 1. List of fungal *P*-type ATPases, used to create a phylogenetic tree in Fig. 4

UniProt	Fungus	Gene name	Protein (aa)	Accession number
Q3MP64	*Candida albicans*	CaJ7.0331	971	AP006852
Q5ABA1	*Candida albicans*	ENA2	1067	AACQ01000036
Q6FM06	*Candida glabrata*	CAGL0K12034 g	1087	CR380957
Q9UUX7	*Neurospora crassa*	Ena-1	1121	AJ243520
Q7S919	*Neurospora crassa*	NCU07966.1	1109	AABX01000219
Q9UUY0	*Neurospora crassa*	nca-1	957	AJ243517
Q9UUX8	*Neurospora crassa*	ph-7	1092	AJ243519
P13587	*Saccharomyces cerevisiae*	ENA1	1091	U24069
P22189	*Schizosaccharomyces pombe*	cta3	1037	J05634
Q4WD68	*Aspergillus fumigatus*	Afu6g03690	1066	AAHF01000012
Q4WPJ1	*Aspergillus fumigatus*	Afu4g09440	1048	AAHF01000005
Q4WIM5	*Aspergillus fumigatus*	Afu2g01320	1079	AAHF01000008
Q5AYI8	*Aspergillus nidulans FGSC A4*	AN6642.2	1066	AACD01000110
Q5BCV2	*Aspergillus nidulans FGSC A4*	AN1628.2	1062	AACD01000026
Q5AVL6	*Aspergillus nidulans FGSC A4*	AN7664.2	1413	AACD01000130
Q5B137	*Aspergillus nidulans FGSC A4*	AN5743.2	972	AACD01000098
Q2UTU3	*Aspergillus oryzae*	AO090009000591	1100	AP007150
Q2UH43	*Aspergillus oryzae*	AO090023000590	1054	AP007157
Q2UMC7	*Aspergillus oryzae*	AO090003000051	1006	AP007155
Q2U8J7	*Aspergillus oryzae*	AO090701000406	1074	AP007164
P78981	*Zygosaccharomyces rouxii*	Z-ENA1	1048	D78567
Q6CJ82	*Kluyveromyces lactis*	KLLA0F20658 g	1082	CR382126
Q6CN82	*Kluyveromyces lactis*	KLLA0E14630 g	1082	CR382125
Q9C1R0	*Debaryomyces hansenii*	ENA1	1076	Af247561
Q96X39	*Debaryomyces hansenii*	ENA2	1073	AF263248
O13397	*Debaryomyces occidentalis*	ENA1	1055	AF030860
O13398	*Debaryomyces occidentalis*	ENA2	1082	AF030861
Q6C172	*Yarrowia lipolytica*	YALI0A01023 g	1078	CR382127
Q6C5C1	*Yarrowia lipolytica*	YAL10E19338 g	1054	CR382131
Q6VAH6	*Fusarium oxysporum f.* sp. *lycopersici*		1087	AY345588

phosphatase motif (DEXLXTGESL), phosphorylation motif (DKTGTLT), and ATP-binding motifs (DPPR, MLTGD and GDGVNXXPSLK) are completely conserved in both of the HwENA amino acid sequences. Like other *P*-type ATPases, both HwENA ATPases contain conserved 10 putative transmembrane regions.

The phylogram based on the amino acid sequences of known fungal ATPases revealed a separate group of ENA fungal ATPases, evolving from a common ancestor with other fungal Na^+ and Ca^{2+} ATPases (Fig. 4a). This group is composed of ENA ATPase from *Aspergillus fumigatus* Af293, NcEna2p and the third NcEna protein of *N. crassa*, both HwEna proteins and putative ENA ATPases from *Aspergillus nidulans FGSC A4* and *Aspergillus oryzae*. All the accession numbers are written in the Table 1. While the function of most of these *P*-type ATPases has not been determined yet, it was speculated that NcEna2p is involved in K^+ metabolism (Benito *et al.*, 2002). A similar function has also been attributed to CTA3 ATPase from *Schizosaccharomyces pombe* (Benito *et al.*, 2002). HwEna1p and HwEna2p, along with other ENA ATPases, possess also several amino acid residues putatively involved in Ca^{2+} transport (Clarke *et al.*, 1989) (Fig. 4b).

Functional analysis of HwENA1 and HwENA2 ATPases

Because fungal $Na^+/K^+$and Ca^{2+} ATPases cannot be distinguished only on the basis of their protein sequences, additional functional analyses are needed. One of the most common used is a functional complementation of studied ENA ATPase in *S. cerevisiae* mutants. The *S. cerevisiae ena1-4nha1* double mutant is very salt sensitive as it lacks all ENA sodium ATPases and plasma membrane Na^+/H^+ antiporter. *HwENA* genes were expressed in such *S. cerevisiae* mutant cells. Drop tests were performed on a series of plates containing increasing concentrations of NaCl (0, 0.25, 0.5, 1.0, 1.5 M) as well as at alkaline pH. *HwENA* genes did not complement ENA function in *S. cerevisiae* mutant cells, neither in neutral nor in the alkaline pH in none of the NaCl concentrations in the media tested. The same results were obtained in the presence of K^+ and Li^+ ions in the media (data not shown). Results were confirmed with the experiments in liquid media with increasing concentrations of NaCl and pH 5, 7 and 8.5. Presented in Fig. 5, only the results of the growth of transformants at pH 5 are shown,

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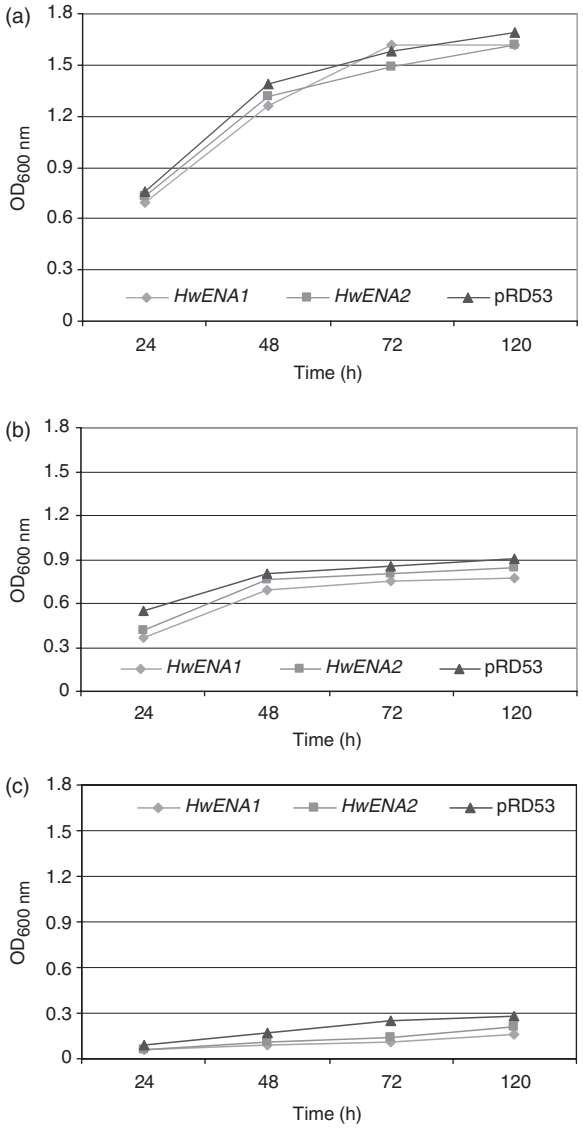

Fig. 5. Growth of *Saccharomyces cerevisiae* BW31a transformants in YNB-Ura+Gal media pH5, expressing *HwENA1* and *HwENA2* in the absence of NaCl (a) and in the presence of 0.25 M NaCl (b) and 0.5 M NaCl (c).

since the cells did not grow at pH 7 and 8.5 in the presence of NaCl. The growth rate of the transformants did not exceed the growth rate of the BW31a mutant with empty vector in whichever media tested. The unsuccessful suppression of the defect of the same mutant was also reported for pH 7, later named NcEna2p (Benito *et al*., 2000), which phylogenetically belongs to the same group of ENA ATPases (see Fig. 4 and Table 1). One can speculate that ENA ATPases from this group are too distinct from *S. cerevisiae ENA1* gene to complement its function.

In conclusion, we present in this paper two novel ENA-like P-ATPases, *HwENA1* and *HwENA2*, isolated from the extremely salt tolerant and adaptable *H. werneckii*. Studies of *HwENA1/2* gene expression revealed that both of them are responsive to the increased salt concentrations and pH and are thus involved in the adaptation mechanisms of *H. werneckii* to increased environmental salinity and alkaline pH, both characteristic for the natural environment of this fungus. The true function of these ATPases is still not clear, since they could not suppress the defects of *S. cerevisiae* BW31 mutant. Phylogenetically, they belong to a separate group of fungal alkali cation P-ATPases, evolving, together with other fungal *P*-type ATPases, from a common ancestor. It is believed, that genes encoding fungal K^+- or Na^+-ATPases (ENA *P*-type ATPases) have most probably evolved from an ancestral K^+-ATPase through the processes of gene duplication and that the capacity of ENA ATPases to pump Na^+ has evolved as an adaptation mechanism to the increased salinity (Benito *et al*., 2002). As revealed by phylogenetic tree, the novel group of fungal *P*-type ATPases is phylogenetically older then fungal Na^+/K^+ ATPases presented in Fig. 4. Therefore it is not surprising that *HwENA* genes, similarly to *NcENA2* from the same group (Benito *et al*., 2000), could not complement classical Na^+ ATPases in *S. cerecisiae* mutant cells. The actual function of the ATPases from this group thus still remains to be established.

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