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Characterization of a Salt-induced *DhSSA* Gene from the Extreme Halophilic Yeast *Debaryomyces hansenii*

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ABSTRACT
Background: *Debaryomyces hansenii* is a highly halotolerant yeast. The objective of this research is to investigate the genes that are involved in *Debaryomyces hansenii* for tolerance mechanisms against salinity. **Methods:** In this experiment, the gene *DhSSA* was cloned from *D. hansenii* and examined for its expression as induced by salt. The *DhSSA* gene is orthologous to the *Saccharomyces cerevisiae SSA* encoded HSP70 which cloned from *D. hansenii* induced by 2.5 M salt stress and significantly up-regulated during salinity stress. The full-length cDNA of *DhSSA* has 2,102 bp of nucleotide and contains a 1,926 bp Open Reading Frame (ORF) encoding a deduced protein of 642 amino acid residues (69.9 kDa). **Results:** Southern blot analysis indicated that *DhSSA* exists in the genome with one or two copy. Semi-quantitative RT-PCR and Real-time PCR results showed that expression of *DhSSA* is rapidly induced by salt and its expression increased with time reaching the highest level at 48 min before decline thereafter. Overexpression of *DhSSA* in *Pichia methanolica* conferred a higher stress tolerance. Immunocytological labeling reveals that *DhSSA* is a cytosolic protein. **Conclusion:** In summary, this study has cloned a salt-induced *DhSSA* gene from the halophilic yeast *D. hansenii* by subtraction hybridization. Functional analysis with overexpression transformants in *P. methanolica* suggest that the gene plays a protective role in the tolerance of *D. hansenii* to stresses.

Key words: *Debaryomyces hansenii*, salinity stress, *DhSSA*, HSP70

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INTRODUCTION
Debaryomyces hansenii is a highly halotolerant yeast. Unlike *Saccharomyces cerevisiae* which is a "sodium excluder", *D. hansenii* is the "sodium includer" yeast[1]. In this species of yeast, NaCl and KCl has similar effect thus, indicating that NaCl created only osmotic effect and specific toxicity of sodium are not involved as has been shown in *S. cerevisiae*. Furthermore, growth of *D. hansenii* was stimulated by NaCl at concentrations up to 1 M NaCl[2]. Presence of salt appeared to stimulate the growth of *D. hansenii* at higher temperature that normally did not support its growth. Moreover, the stress effect of extreme pH had been shown to be relieved by the presence of 0.25 M NaCl[3]. The heat shock response is widely recognized and accepted as a major weapon in the cell's armamentarium for protection against and recovery from environmental insult, both physical and chemical[4,5]. One of the salt-induced genes, *DhSSA*, is orthologous to the *S. cerevisiae SSA* encoded HSP70. HSPs, particularly the members of the HSP70 family, one of the most highly conserved proteins in the cell, could be induced under stress conditions, so the goal was to get a view as complete as possible of *DhSSA* gene involved in the response of *D. hansenii* to salinity.

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MATERIALS AND METHODS
Yeast strains and growth conditions: The yeast strains used in this study included a *D. hansenii* strain BCRC No. 21947, isolated from Hsilo County, Taiwan and obtained from FIRDI (Food Industry Research and Development Institute, Hsin-chu City, Taiwan). A *Pichia methanolica* strain, PMAD11 genotype *ade2-11*, was obtained from Invitrogen, U.S.A. The *D. hansenii* strain was cultured at 24°C in a YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose) while *P. methanolica* was cultured at 28EC in a YPAD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.01% adenine), respectively.

Cloning and sequencing of *DhSSA* from *D. hansenii*: For screening up-regulated genes, subtractive hybridization was performed using PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) and

D. hansenii was inoculated at YM11 medium overnight, then adding NaCl to the medium to a final concentration of 2.5 M NaCl, after 24 min, total RNA was extracted. The full-length *DhSSA* from *D. hansenii* was obtained by rapid amplification of the cDNA ends using the GeneRacerTM Kit (Invitrogen, USA), following the manufacturer's instructions. After checking the sequences of the cloned PCR fragments using an automatic DNA sequencer (3100 Genetic Analyzer, ABI, U.S.A). Alignment and phylogenetic analysis were carried out using the protein sequence alignment program CLUSTAL W.

Southern hybridization analysis: Southern hybridization analysis was performed using the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Diagnostics, Switzerland). DNA (20 µg) was digested with restriction endonuclease *Eco*RI, *Bam* HI and *Himd* III, respectively, then fractionated in a 0.7% (w/v) agarose gels and blotted onto a nylon membrane (Amersham Pharmacia Biotech, UK) and UV cross-linked.

Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Total RNA isolated from yeast cells treated with NaCl for various time intervals, extracted with Trizol reagent (Invitrogen, U.S.A.), treated with DNase (RQ1 RNase-free DNase, Promega, U.S.A.), purified using phenol: Chloroform: Isoamyl alcohol (25:24:1,v:v:v) and chloroform: Isoamyl alcohol (24:1) and stored at -80°C. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (P/N 4368814, ABI, USA) for RT-PCR according to the manufacturer's instruction. Serial changes of target gene expression were evaluated using a semi-quantitative RT-PCR technique with the *D. hansenii* ribosomal RNA primers was used as a control. PCR was performed with the following cycling conditions: 1 cycle at 94°C for 3 min, 25 cycles at 94°C for 1.5 min, 59°C for 1.5 min and 72°C for 1.5 min, followed by 72°C for 10 min and a 4°C hold. The following forward and reverse primers were used:

- **Internal F:** 5'-CGTCCCTGCCCTTTGTACAC-3'
- **Internal R:** 5'-GCCTCACTAAGCCATTCAA TCG-3'
- ***DhSSA* F:** 5'TTTAGGGACCACCTACTCGTG TGT3'
- ***DhSSA* R:** 5' CTCTTGGAGCTGGTGGAA TACCT 3'

Quantification of *DhSSA* gene expression by real-time RT-PCR: Total RNA was isolated from yeast cells as described above for Semi-quantitative RT-PCR. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop, Wilmington, USA). This experiment was carried out using an ABI PRISM 7500 Sequence Detection System (ABI, U.S.A.) using SYBR-Green chemistry. The sequence forward and reverse primers for qRT-PCR were designed using the primer Express[R] Software provided by Applied Biosystems. Each sample was conducted in triplicate. The relative expression was calculated as 2-ÎÎCt; Ct: Cycle threshold. Data presented were means +/-SD from 3-4 replicates of measurement.

The following forward and reverse primers were used:

- **Internal F:** 5'-CGTCCCTGCCCTTTGTACAC-3'
- **Internal R:** 5'-GCCTCACTAAGCCATTCAAT CG-3'
- ***DhSSA* F:** F5'-GCTATGAATCCAGCTAATACT GTT-3'
- ***DhSSA* R:** F5'-GGAAATGTTTGATATCACCT TGA-3'

Expression of P_{AUG1}/*DhSSA*/V5/His fusion protein and western blotting: Total proteins were extracted from yeast cell pellets, using the Total Protein Miniprep Purification Kit (GMbiolab Co, Ltd.). Thirty micrograms of protein from the transformants of *P. methanolica* were loaded onto a 12% polyacrylamide gel and electrophoresed for Western blot analysis. Then, the proteins were transferred to a Hybond TM-P membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) and immunostained. It was used anti-V5 antibody to confirm the presence of *DhSSA*/V5/His protein extracted from the transformants of *P. methanolica*.

Overexpression of *DhSSA* in *P. methanolica*: The growth of wild types and transformants yeast were test under different condition of solid media containing 0.5% methanol and 1.0 M LiC concentrations than they can normally tolerate. On the other hand, a single colony of wild types and transformants of *P. methanolica* strain were inoculated for 37 °C temperature stress analysis test.

***DhSSA*/V5/His recombinant protein and immunofluorescent staining:** The transformed yeast cells were cultivated and fixed in 4% formaldehyde for 24 h and then washed. Spheroplasts were prepared by treating the suspension cells with lyticase (0.9 mg mLG^{1}) in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1.2 M sorbitol to break down yeast cell wall. After transfection and dehydration with 100% cold methanol for 10 min, spheroplasts were spotted on

polylysine-coated slides and incubated sequentially with anti-V5 antibody (Invitrogen, U.S.A.) and then incubated in PBS for 1 h with a FITC-conjugated sheep antimouse antibody (1:100; Cappel), FM4-64 (Invitrogen, USA) and DAPI (Sigma, USA). After cells were washed four times in PBS, immunofluorescence was measured using a confocal microscope (TCS-SP5, Leica, Germany).

RESULTS

Cloning and sequencing of *DhSSA* from *D. hansenii*: Because the HSP70 protein is coded by the *SSA* gene in *S. cerevisiae*, searched for the possible existence of an orthologous gene in *D. hansenii*. It was used primers designed from conserved sequences of the *SSA* gene family to identify the orthologous gene in *D. hansenii*. A single DNA fragment of about 1.5 kb (Fig. 1a) was amplified using the primers of GeneRace 3' and forward GSP. According to the 3'-end fragment sequence, a specific reverse (GSP) was designed to amplify the 5'-end and a fragment of 1.5 kb was obtained (Fig. 1b). The alignment of the 3' and 5' RACE products shows that the full-length cDNA of *DhSSA* has 748 bp overlapped. Full-length cDNA of *DhSSA* gene includes 2,102 bp and has an Open Reading Frame (ORF) of 1,926 bp, with 53 bp and 96 bp segments corresponding to the 5' and 3' untranslated regions (Fig. 2). The *DhSSA* gene encodes 642 amino acids with a theoretical molecular mass of 69.9 kDa and pI of 4.94.

Eleven protein sequences from the GenBank database that encode *SSA*s, called *SSA*1-*SSA*4, were analyzed in yeast (Table 1). *DhSSA* protein sequence results suggest that the percentage of identical protein sequences with other yeast's *SSA* family ranged from 80-90%. The highest percentage of similarity appeared in *C. albicans SSA*1 (90%), *C. albicans SSA*2 (90%) and *L. elongisporus SSA*2 (90%), followed by *L. elongisporus SSA*2 (86%), *S. cerevisiae SSA*1 (85%) and *S. cerevisiae SSA*2 (85%). The lowest score appeared in *S. cerevisiae SSA*4 (80%). The isolated gene is homologous to the *SSA1* and *SSA2* genes of *S. cerevisiae* and was therefore called *DhSSA*.

The phylogenetic tree in Fig. 3 shows that the *SSA* protein family includes three major groups: *DhSSA*, *L. elongisporus SSA*2 and *C. albicans SSA*2 form the upper part of the tree while *S. pombe SSA*1, *S. pombe SSA*2, *L. elongisporus SSA*1, *C. albicans SSA*1, *C. albicans SSA*4, *S. cerevisiae SSA*3 and *S. cerevisiae SSA*4 form a major group in the middle of the tree and *S. cerevisiae SSA*1 and *S. cerevisiae SSA*2 form a lower group. This phylogenetic tree places *L. elongisporus SSA*2 and *C. albicans SSA*2 in one cluster, *S. pombe SSA*1 and

Table 1: Percentage of protein sequence identity between *DhSSA* and *SSA* families

Species	*SSA*1	*SSA*2	*SSA*3	*SSA*4
Schizosaccharomyces pombe	82	82	-	-
Lodderomyces elongisporus	86	90	-	-
Candida albicans	90	90	-	84
Saccharomyces cerevisiae	85	85	81	80

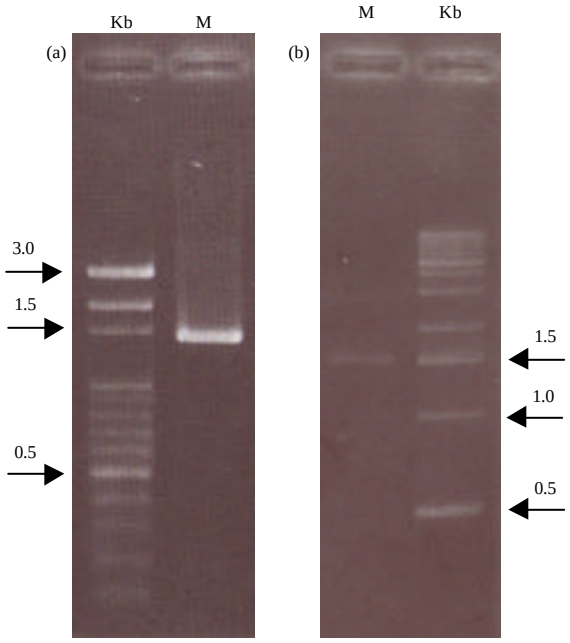

Fig. 1(a-b): Gel analysis of the (a) *DhSSA* 5'-end and (b)3'-end amplification products from *D. hansenii*. M: DNA markers

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5'gtataatagactattaaattatctattaattaagaatttatataaaataaacc
1 atgtctaaagctgttggtattgatttagggaccacctactcgtgtgtagcccatttttct
 M S K A V G I D L G T T Y S C V A H F S
61 aatgatagagttgaaattattgctaatgatcaaggtaatagaactactccatcatttgtt
 N D R V E I I A N D Q G N R T T P S F V
121 ggatttactgatactgaaagattaattggtgatgctgctaaaaatcaagctgctatgaat
 G F T D T E R L I G D A A K N Q A A M N
181 ccagctaatactgttttcgatgctaaacgtttaatcggaagaaaatttgatgatagtgaa
 P A N T V F D A K R L I G R K F D D S E
241 gttcaaggtgatatcaaacatttcccatttaaagttgttgaaaaatccggtaaacctcat
 V Q G D I K H F P F K V V E K S G K P H
301 attcaagttgaatataaaggagaagataaagttttcactccagaagaaatttctttctatg
 I Q V E Y K G E D K V F T P E E I S S M
361 gttttaggtaaaatgaaagaaactgctgaaggtttcttaggtactaaagttaatgatgct
 V L G K M K E T A E G F L G T K V N D A
421 gttgttaccgttccagcttatttcaatgattctcaaagacaagctaccaaagatgctggt
 V V T V P A Y F N D S Q R Q A T K D A G
481 ttaattgctggtttaaacgttttaagaatcattaacgaacctactgctgctgccattgct
 L I A G L N V L R I I N E P T A A A I A
541 tatggtttagataaaaaagatgaacaagaaaagaatgttttaattttcgatttaggtggt
 Y G L D K K D E Q E K N V L I F D L G G
601 ggtactttttgatgtctctttattatctattgaagatggtatctttgaagttaaatctact
 G T F D V S L L S I E D G I F E V K S T
661 gctggtgatactcacttgggtggtgaagatttcgatcatagattagttaaccatttcatt
 A G D T H L G G E D F D H R L V N H F I
721 aatgaatttaagagaaaaaacaagaaagatatttctaccaatcaaagagctttaagaaga
 N E F K R K N K K D I S T N Q R A L R R
781 ttaagaactgcttgtgaaagagctaagagaactttatcttcctctgctcaaacttctatt
 L R T A C E R A K R T L S S S A Q T S I
841 gaaattgattctttatatgaaggtattgatttctacacttctatcaccagagctagattt
 E I D S L Y E G I D F Y T S I T R A R F
901 gaagaattatgtgctgatttattcagatctaccatcgacccagttgaaagagttttaaga
 E E L C A D L F R S T I D P V E R V L R
961 gatgctaaagttgataaatctcaagttcatgaaatcgtcttagttggtggttctaccaga
 D A K V D K S Q V H E I V L V G G S T R
1021 atcccaaaaattcaaaaattagtatctgacttcttcaatggtaaagaaccaaacagatct
 I P K I Q K L V S D F F N G K E P N R S
1061 attaacccagatgaagccgttgcttatggtgctgctgttcaagctgctatcttaactggt
 I N P D E A V A Y G A A V Q A A I L T G
1121 gatgaatcttctaaaactcaagatttattattattagatgttgctccattatctttaggt
 D E S S K T Q D L L L L D V A P L S L G
1161 attgaaactgctggtggtatcatgactaaattaattccaagaaactccactattccaacc
 I E T A G G I M T K L I P R N S T I P T
1221 aaaaaatcagaaacttttctctacttatgctgataaccaaccaggtgtcttaattcaagta
 K K S E T F S T Y A D N Q P G V L I Q V
1281 tttgaaggtgaaagagctaaaactaaggataacaacttattaggtaagtttgaattatcc
 F E G E R A K T K D N N L L G K F E L S
1341 ggtattccaccagctccaagaggtgttccacaaattgaagttactttcgatatggatgct
 G I P P A P R G V P Q I E V T F D M D A
1401 aatggtatcttgaatgtttctgctgttgaaaaaggtactggtaaaactcaacaaattact
 N G I L N V S A V E K G T G K T Q Q I T
1461 attaccaacgataaaggtagattaagtaaagaagatattgaaagaatggttaatgaagct
I T N D K G R L S K E D I E R M V N E A
1521 gaaaaattcaaagaagaagatgaaaaagaagctaacagaatccaagctaaaaacggttta
 E K F K E E D E K E A N R I Q A K N G L
1581 gaatcttatgcttactccttaaaatctactttaggtgaagaacaaatgaaagaaaaatta
 E S Y A Y S L K S T L G E E Q M K E K L
1601 gatgctgctgaaattgaagaagttaccaaagctgctgatgaaactattgaatggttagat
 D A A E I E E V T K A A D E T I E W L D
1661 gctaaccaaactgctgctcaagacgaattcgctgacaaacaaaaagaattagaaggtaaa
 A N Q T A A Q D E F A D K Q K E L E G K
1721 gctaacccaatcatggctaaagcttaccaagctggtggtgctccaggtggaatgccaggt
 A N P I M A K A Y Q A G G A P G G M P G
1781 gctgctcctggtgctggtgctgctccagatgcttctaatgatggtccaactgtcgaagaa
 A A P G A G A A P D A S N D G P T V E E
1841 gttgattaa
 V D -
gggtgattcttgtagtataattcacgaggaacttagattatataatgatagttatagaattag
agttatatagagtaataaagaaataggtatattaaaaaaaaaaaaaaaaaaaaaaaa 3'
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Fig. 2: Full-length nucleotide sequence of *DhSSA* and the predicted amino acid sequence of its protein. The start and stop codons ATG and TAA were boxed

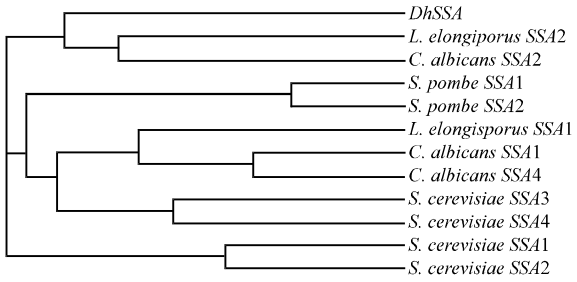

Fig. 3: Phylogenetic relationship between *SSA* of *D. hansenii* and *SSA* proteins from other yeast

*S. pombe SSA*2 in another cluster, *C. albicans SSA*2 and *C. albicans SSA*4 in another cluster, *S. cerevisiae SSA*3 and *S. cerevisiae SSA*4 in another cluster, *S. cerevisiae SSA*1 and *S. cerevisiae SSA*2 in another cluster and *DhSSA* and *L. elongisporus SSA*1 on their own. These results reveal the evolutionary relationship of *SSA* proteins in yeast, suggesting that the *SSA* of *D. hansenii* is closely related to that in yeasts. It is conceivable that its function or characteristics may be close to those of yeast *SSA*s.

Genome organization and expression of *DhSSA*: To examine the distribution of the *DhSSA* in the *D. hansenii* genome. Southern blot analysis was performed using the full-lengthed *DhSSA* DNA as a probe. As shown in Fig. 4a-b *DhSSA* hybridized with only one genomic fragment in two different restriction digestions (*Eco*R I, *Bam*H II), in addition to, with two genomic fragment in *Himd*III restriction digestions, indicating that *DhSSA* exists as a one and two -copy gene in the *D. hansenii* genome.

Expression of *DhSSA* measured by semi-quantitative RT-PCR and qRT-PCR: Gene expression was examined to determine whether *DhSSA* exhibited salt regulation within *D. hansenii*. In order to understand the expression of *DhSSA* in *D. hansenii*, initially applied RT-PCR and qRT-PCR for our research. As shown in Fig. 5, *DhSSA* was present in *D. hansenii* treated with NaCl for various time intervals with the highest abundance in 48 min. qRT-PCR showed the similar results as semi-quantitative RT-PCR. A large increase in *DhSSA* transcript was detected as early as 12 min upon salt treatment but its expression was rapidly accelerated thereafter. Its level increased 15.1, 31.3 and 7.6 fold after 24, 48 and 72 min of induction, respectively. Therefore, the transcript reached the peak after 48 min and it dropped off at 72 min (Fig. 6).

Expression of P_{AUG1}/*DhSSA*/V5/His fusion protein and western blotting: The white colonies on MD plate were picked to verify Mut^+ (methanol utilization plus)

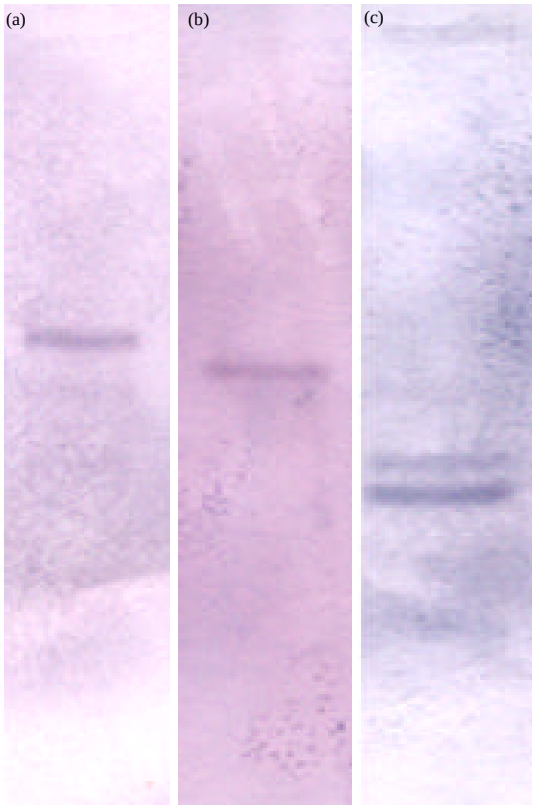

Fig. 4(a-c): Southern hybridization analysis of *D. hansenii* genomic DNA with probes from *DhSSA*. DNA samples were digested with different restriction enzymes (a) *Eco*RI, (b) *Bam*HII and (c) *Himd*III

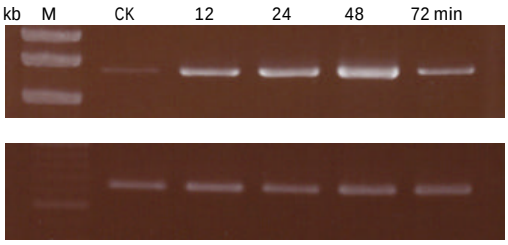

Fig. 5: Expression of *DhSSA* cDNA with 2.5 M NaCl treatment at 12, 24, 48 and 72 min in *D. hansenii* by semi-quantitative RT-PCR analysis (a) *DhSSA* and (b) 18S

and Mut^S (methanol utilization slow) transformant colonies of *P. methanolica*. The transformants were selected for a*SSA*ying expression fusion protein expression by dot blotting using anti-V5 antibody firstly.

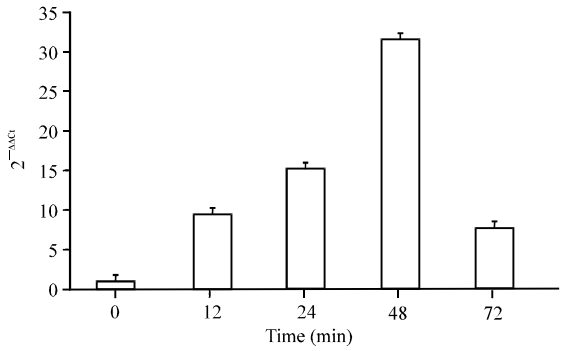

Fig. 6: Time course of induction of *DhSSA* by 2.5 M NaCl, as determined by Real-Time RT-PCR. Its expression level increased 9.3, 15.1, 31.3 and 7.6 fold after 12, 24, 48 and 72 min of induction, respectively. Data presented were Mean ± SD from 3-4 replicates of measurement

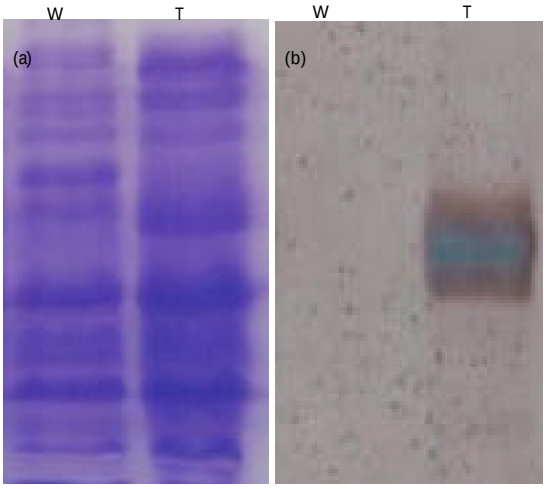

Fig. 7(a-b): (a) Total protein extracted from yeasts after inoculation for 72 h and separated by 12% SDS-PAGE and (b) *DhSSA*/V5/His protein confirmed by western blotting using anti -V5 antibody and W: wild type; T: transformant

And then the transformants were confirmed having good expression of the fusion protein which had a V5 epitope in its C-terminal region by western blotting that a single positive band at about 69.9 kDa appeared (Fig. 7).

Overexpression of *DhSSA* in *P. methanolica*: The growth of *DhSSA* transformants in *P. methanolica* appeared better than the wild type (Fig. 8a). In the presence of 1.0 M LiC,l wild type strain could not grow while the transformant could grew. The degrees of enhancement in

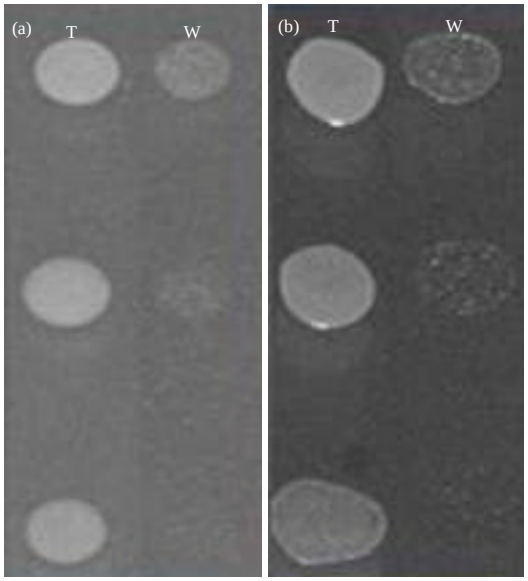

Fig. 8(a-b): Growth of wild type and transformant of *P. methanolica* on YPAD medium with 0.5% methanol. (1 × suspension: $O.D_{600}$ = 0.3) W: Wild type, T: Transformant (a) YPAD + 1.0 M LiCl and (b) 37°C

LiCl tolerance by overexpression of *DhSSA* was significant in *P. methanolica*. The results indicate that overexpression of *DhSSA* confers enhanced LiCl tolerance in *P. methanolica*, allowing them to be able to grow at higher LiCl levels than they can normally tolerate.

Furthermore, under high temperature (37°C), growth of wild type and transformants were different (Fig. 8b). However, the growth of transformants were clearly increased drastically under 37°C. The results indicate that overexpression of *DhSSA* confers enhanced stress tolerance in *P. methanolica*, allowing it to be able to grow at higher srtress levels than they can normally tolerate.

***DhSSA*/V5/His recombinant protein and immunofluorescent staining:** To determine the subcellular distribution of *DhSSA*/V5/His, recombinant protein was expressed in the *P. methanolica* strain PMAD16. Figure 9 presents these results, showing that *DhSSA*/V5/His recombinant protein appeared in the cytoplasm. However, the control experiment, where the transformant was not induced by methanol for the expression of the fusion protein, revealed no staining of the recombinant protein.

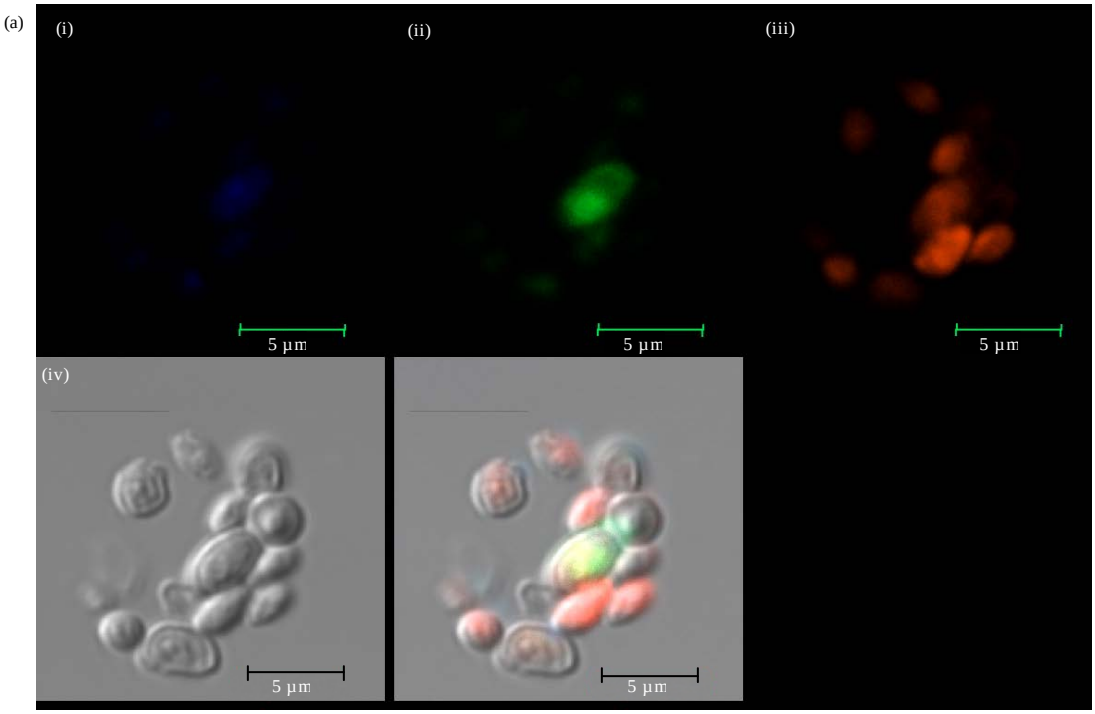

Fig. 9(a-b): Continue

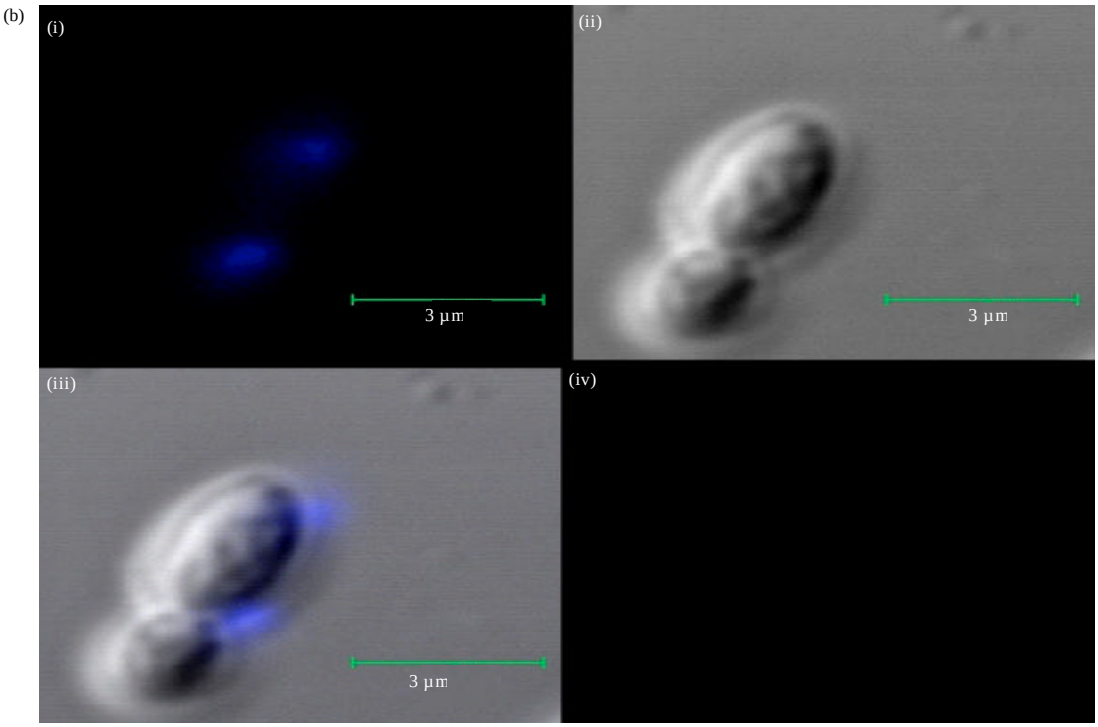

Fig. 9(a-b): Localization of *DhSSA* in *P. methanolica* by confocal microscopy, (a) *DhSSA*/pMETB transformant and (b) Untransfected *P. methanolica*, (a) Yeast cells transfected with *DhSSA*/pMETB plasmids were fixed and prepared for immunocytochemical study. The V5 antibody was used to detect *DhSSA* fusion protein. Alexa fluor-conjugated antibodies track *DhSSA* protein (green, ii) DAPI staining of DNA was used to localize the nucleus (blue, i), FM4-64 staining of lipid was used to localize plasma membrane (red, iii) and (iv) light field, (b) Untransfected *P. methanolica* as a negative control

DISCUSSION

When *D. hansenii* exposed to salinity for 24 min, a considerable number of genes were either up-regulated or down-regulated, implying that adaptation or tolerance to salt exposure is a complex process involving rapid up-regulation or down-regulation of sets of genes, many of unknown function. Chen *et al.*[6] indicated that the response to high salinity involves the coordinated induction of transcription in many genes, thus requiring the activity of specific sets of transcription factors and their binding to specific sequences in the promoter regions of target genes. Therefore, the information derived from subtractive hybridization studies of *D. hansenii* exposed to salinity should facilitate identification of specific promoters for the control of gene expression in response to salinity.

Due to HSPs' responsiveness to diverse forms of stress, heat shock response has been widely used in biomonitoring and environmental toxicology applications [7, 8]. Compared to other classes of heat shock proteins, the HSP70 family proteins are one of most highly conserved and first to be induced under stress conditions. Cells respond to protein-denaturing stresses by rapidly inducing the expression of a wide array of heat shock genes. One of the salt-induced genes, *SSA*, is orthologous to the *S.cerevisiae SSA* encoded HSP70. The *SSA* subfamily, in yeast consists of four members (*SSA1-4*). During growth, only *SSA1* and *SSA2* are expressed at high levels, whereas *SSA3* and *4* are not. The most well-studied Hsp 70 sec in yeast are the cytoplasmic *SSA* proteins which are encoded by the differentially expressed *SSA1–4* genes. *SSA*1–4 performs largely redundant functions and the presence of at least one *SSA* gene is required for viability [9]. The HSPs are an evolutionarily conserved family that is ubiquitous in nature and exerts many functions in protein synthesis, transport, maintenance and degradation. As a result, HSPs have been called "molecular chaperones"[10]. Under various stress conditions, the synthesis of stress-inducible HSP70 enhances the ability of stressed cells to cope with increased concentrations of unfolded proteins[11]. The overexpression of HSP70 can protect cells from

stress-induced apoptosis[12]. Due to its responsiveness to diverse forms of stress, heat shock response has seen wide spread application in biomonitoring and environmental toxicology[7, 8].

The results of this study reveal that *DhSSA* is induced at higher levels by salinity from *D. hansenii* and reported for the first time in this study (Fig. 5,6). HSPs also stabilize several cellular processes, such as transcription, splicing, translation and transport[13]. Transcripts of *DhSSA* accumulated in the stressed *D. hansenii* after exposure to salt for 24 min. Quantitative analysis of the time course changes in *SSA* gene expression by real-time PCR confirmed that the induction of *SSA* reached its maximum of 31.3 fold at 48 min. Consistent with the semi-quantitative RT-PCR results, we can confirm patterns of *DhSSA* gene expression was induced by salt and maximum induction at 48 min (Fig. 5-6).

The experiments in this study expressed the *DhSSA*/V5/His recombinant protein using the *P. methanolica* yeast expression system in a location dependent functional study. The use of the highly efficient *AUG1* gene promoter is an important feature in the suitability of *P. methanolica* as a host system for heterologous expression. This set of functions suggests that *DhSSA*/V5/His recombinant protein should be located in the cytosol (Fig. 9). HSP70 is an ATP-dependent molecular chaperone because it can protect nascent or denatured protein from aggregation and assist its folding or refolding its native conformation[14]. HSP70 is one of the most abundantly-induced proteins under a variety of stress conditions[15]. Therefore, *DhSSA* gene expression can lead largely by salt and protects essential proteins from denaturation in the yeast's adaptation to salinity.

In conclusion, this study investigates *DhSSA* gene generated by subtractive hybridization analyse of *D. hansenii* subjected to salinity. The forward cDNA subtractive hybridization analyses in this paper suggest that the creation of high salt concentration stress-tolerant *D. hansenii* is to enhance heat shock proteins. Real-time PCR results indicate that HSP70 may be part of a general stress response even though its induction occurs shortly after imposition of salinity conditions. In addition, HSP70 plays a key role in the stress tolerance of transformants of *P. methanolica*. The results of this study help explain the complex regulatory mechanisms associated with salinity responses in *D. hansenii*.

REFERENCES

1. Prista, C., M.C. Loureiro-Dias, V. Montiel, R. Garcia and J. Ramos, 2005. Mechanisms underlying the halotolerant way of *Debaryomyces hansenii*. FEMS Yeast Res., 5: 693-701.
2. Prista, C., A. Almagro, M.C. Loureiro-Dias and J. Ramos, 1997. Physiological basis for the high salt tolerance of *Debaryomyces hansenii*. Applied Environ. Microbiol., 63: 4005-4009.
3. Almagro, A., C. Prista, S. Castro, C. Quintas, A. Madeira, J. Ramos and M.C. Loureiro-Dias, 2000. Effects of salts on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under stress conditions. Int. J. Food Microbiol., 56: 191-197.
4. Georgopoulos, C. and W.J. Welch, 1993. Role of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol., 9: 601-634.
5. Chao, H.F., R. Prasenmool and Y.F. Yen, 2013. Cloning and overexpression of a DhIFN-"1 gene from the halophilic yeast of *Debaryomyces hansenii* into *Pichia methanolica* to enhance its tolerance to salt and temperature stresses. Sci. Int., 1: 222-229.
6. Chen, X., T. Kanokporn, Q. Zeng, T.A. Wilkins and A.J. Wood, 2002. Characterization of the V-type $H^{(+)}$-ATPase in the resurrection plant Tortula ruralis: Accumulation and polysomal recruitment of the proteolipid c subunit in response to salt-stress. J. Exp. Bot., 53: 225-232.
7. Ryan, J.A. and L.E. Hightower, 1996. Stress proteins as molecular biomarkers for environmental toxicology. EXS., 77: 411-424.
8. Sanders, B.M. and S.D. Dyer, 1994. Cellular stress response. Environ. Toxicol. Chem., 13: 1209-1210.
9. Werner-Washburne, M., D.E. Stone and E.A. Craig, 1987. Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol., 7: 2568-2577.
10. Cabiscol, E., G. Belli´, J. Tamarit, P. Echave, E. Herrero and J. Ros, 2002. Mitochondrial Hsp60, resistance to oxidative stress and the labile iron pool are closely connected in *Saccharomyces cerevisiae*. J. Biol. Chem., 277: 44531-44538.
11. Nollen, E.A., J.F. Brunsting, H. Roelofsen, L.A. Weber and H.H. Kampinga, 1999. *In vivo* chaperone activity of heat shock protein 70 and thermotolerance Mol. Cell. Biol., 19: 2069-2079.
12. Didelot, C., E. Schmitt, M. Brunet, L. Maingret, A. Parcellier and C. Garrido, 2006. Heat shock proteins: Endogenous modulators of apoptotic cell death. Handb. Exp. Pharmacol., 172: 171-198.
13. Vega, V.L., W. Charles and A. De Maio, 2010. A new feature of the stress response: increase in endocytosis mediated by Hsp70. Cell Stress Chaperones., 15: 517-527.
14. Heikkila, J.J., 2010. Heat shock protein gene expression and function in amphibian model systems. Comp. Biochem. Physiol. Part A Mol. Integrative Physiol., 156: 19-33.
15. Lachapelle, G., S.M. Radicioni, A.R. Stankiewicz and D.D. Mosser, 2007. Acute acidification or amiloride treatment suppresses the ability of Hsp70 to inhibit heat-induced apoptosis. Apoptosis, 12: 1479-1488.