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SUMMARY OF THE THESIS

Living organisms (from simple unicellular to complex multicellular) have to survive a variety of stresses prevalent on earth. To increase the scope of their survival, the organisms surmount various adaptive responses and acquire levels of tolerance to the extreme environmental conditions. Over the last few years, extremophiles have become a focus of increasing scientific attention, with some novel discoveries regarding the mechanism of stress tolerance emanating from these efforts. These extremophiles - both prokaryotes as well as eukaryotes- are being perceived as promising models to strengthen our understanding of the functional evolution of stress adaptation. Among these, halotolerant quality of yeast *D. hansenii* has triggered many investigations to understand the cellular mechanisms involved in salt dependent growth regimes. The present study has been focused on *DhPPZ1* which encodes a regulatory component of the signal transduction pathways involved in salt tolerance in *D. hansenii*. In *S. cerevisae*, Ppz1p (692 residues) and Ppz2p (710 residues) represents a unique and novel phosphatase that are ubiquitous in fungi. A TBLASTN search through *D. hansenii* genome database revealed a single 572 amino acid long hypothetical protein (at chromosome E) as putative *PPZ* phosphatase which showed 54.8% and 54.2% identity with Ppz1p and Ppz2p of *S. cerevisae* respectively. Since it is the first *PPZ* ortholog from *D. hansenii*, it was designated as *DhPPZ1*. Interestingly, the N-terminal half of DhPpz1p exhibited only 25.0% and 35.4% identity with that of Ppz1p and Ppz2p whereas C-terminal catalytic region of DhPpz1p showed 85.2% and 83.4% identity with that of Ppz1p and Ppz2p respectively. The presence of N-myristoylation site (a prominent feature of Ppz phosphatase) has also been predicted *in silico* in Ppz1p, Ppz2p and DhPpz1p with 0.995008, 0.995168 and 0.995024 positive

scores respectively. Thus, it appeared that *DhPPZ1* is not a typical ortholog of either *PPZ1* or *PPZ2* of *S. cerevisae*, rather shares equal sequence identity with both the proteins.

The involvement of *DhPPZ1* in salt tolerance pathway could not be confirmed fully by functional complementation in *S. cerevisae* mutants. So to characterize it further in its native host, work on the development of methods for genetic manipulation in *D. hansenii* was carried out. The transformation system for *D. hansenii* was developed based on a histidine auxotrophic recipient strain and a replicative plasmid (pDH4) harbouring *C. famata* ARS element (CfARS16). A stable histidine auxotroph DBH9 was isolated using UV mutagenesis protocol. Transformation protocol was standardized with some modification in the *C. famata* protocol (Andriy *et al.*, 2002). Successful transformation thus indicated that CfARS16 also functions well in *D. hansenii*. RFP gene expression in DBH9 transformed with expression plasmid pDH11 (with *RFP* (DsRedT.4) as reporter gene under the regulation of the putative promoter of *DhTEF* gene) also validated the successful functioning of the transformation system and expression plasmid. Several autonomous replication sequences (ARS) were also isolated by screening the *D. hansenii* genomic library. Among these, two plasmids pDhARS2 (bearing DhARS2 element) and pDhARS9 (bearing DhARS9 element) were characterized further. They exhibited high transformation efficiency with structural stability. The present work also showed for the first time a successful attempt of gene disruption in *D. hansenii* using xylose reductase (*DhXR*) gene through homologous recombination. Furthermore, using the same UV mutagenesis protocol, a stable double auxotroph (DBH93) which required histidine and arginine for their growth in SD

minimal media was also isolated. Since this strain carried two auxotropic markers it would very useful for in vivo structure function analysis of a *D. hansenii* protein.

For characterizing *DhPPZ1* gene further in its native host, *D. hansenii* mutant strains (DBH91 and DBH936) carrying a deletion in *DhPPZ1* gene were generated using homologous recombination. The role of *DhPPZ1* in the halotolerance in *D. hansenii* was confirmed by observing prominent growth of DBH91 at 1M LiCl compared to the parent strain. The same was also observed for DBH91 in liquid medium, at all concentrations of LiCl tested. Regarding NaCl tolerance, on solid media, little or no improvement in the growth of the mutant was observed however in liquid media, the mutant showed a better growth at lower NaCl concentration compared to DBH9. Further results also confirmed that the salt tolerance exhibited by Δ*dhppz1* strain was dependent on the pH of the growth medium. In addition to LiCl tolerance, DBH91 also exhibited improved growth on high concentration of other toxic cations (hygromycin and spermine) compared to the parent strain. To make a detailed insight into the mechanism of halotolerance in Δ*dhppz1* strain, expression of DhEna1p, DhTrk1p and DhNha1p transporter was compared in Δ*dhppz1* mutant and parent strain. Of these transporters, no significant effect on the expression level of *DhTRK1* was observed whereas *DhENA1* gene showed up-regulated expression in DBH91 only under alkaline conditions. However, *DhNHA1* gene showed higher level of expression in both alkaline and LiCl treated conditions. Further, DBH936 (Δ*dhppz1*) strain found accumulating lower steady state concentration of internal lithium and higher concentration of internal potassium compared to DBH93 (parent) strain. FACS results further showed that DBH936 exhibited increased cell size compared to parent strain, which was in accordance with the observed raise in K^+ ion content. These results thus

indicated regulation of the activity of various transporters e.g. DhTrk1p by DhPpz1p. In earlier studies carried in *S. cerevisiae,* regulation of Trk1p by Ppz1p was postulated as they were shown to be localized in the plasma membrane in close proximity. However, the picture is quite different in *D. hansenii*. As a transporter DhTrk1p is present in membrane. Our result had clearly confirmed cytosolic location of DhPpz1p and therefore the mode of direct interaction, if any, is unlikely to be similar. The vital role of DhPpz1p in cell growth was evident from the slow growth exhibited by Δ*dhppz1* mutant and moreover, the over-expression of *DhPPZ1* had negligible effect on growth of *D. hansenii*. In these aspects also, physiological role of DhPpz1p in *D. hansenii* was very distinct from its *S. cerevisiae* orthologs.

In *D. hansenii*, Δ*dhmpk1* strains (DBH932 and DBH933) and Δ*dhppz1* strains (DBH91 and DBH936) also showed cell lysis defect at 32°C compared to parent strain, which could be rescued by addition of 1M sorbitol in to the medium. These mutant strains also exhibited sensitivity toward caffeine at normal growth temperature. However, suppression of caffeine sensitivity of Δ*dhmpk1* strains by over-expression of *DhPPZ1* and up-regulated transcript levels of *PIR3* and *SED1* (targets of the Mpk1 pathway) in Δ*dhppz1* strains, suggested some kind of genetic and functional linkage between *DhPPZ1* and cell wall integrity pathway in *D. hansenii*. Δ*dhmpk1* mutant also exhibited slow growth pattern quite similar to Δ*dhppz1*, which was restored to normal by expressing *DhPPZ1* under strong promoter (pAN6). Δ*dhmpk1* strain harbouring pAN6 plasmid also showed remarkable lithium resistance compared to parent strain. However, no change in the hygromycin sensitivity of this mutant strain was observed with either pAN5 or pAN6 plasmid. These results indicated that over expression of

DhPpz1p only improved the growth and lithium sensitivity specific phenotype of Δ*dhmpk1* strain.

Like a typical PPZ ortholog, DhPpz1p consisted of a conserved, catalytic COOH-terminal region and a divergent, regulatory NH_2-terminal region rich in serine and asparagine residues. Both the regions are essential for its function as evidenced from the phenotypic analysis. Analysis of the primary sequence of NH2-terminal region (1-278 aa) of DhPpz1p with SMART showed six regions of low complexity that are rich in serine or asparagine residues. The role of these regions in the functionality of DhPpz1p was thus explored. Deletion of any of these regions did not affect the caffeine sensitivity and thus these were not important for DhPpz1p towards cell wall or cell cycle regulation. In contrast, the region corresponding to 27-36 amino acid residues are very important for both lithium and hygromycin tolerance phenotype while 106-120 amino acid residues is involved only in lithium tolerance. From sequence alignment it was apparent that the residues 106-120 partially overlapped with a highly conserved region only in Ppz1p, Ppz2p and DhPpz1p. Therefore, the deletion of 106-120 residues might have affected its function. However, the 27-36 amino acid motif appeared to be highly conserved in orthologs from several species including those from *S. cerevisiae*. This motif is rich in serine and arginine residues with a consensus S-[STG]-R-S-X-R-S-X (2)-S sequence.

To understand regulatory role of DhPpz1p and identify the pathways that are affected by deletion of *DhPPZ1* in *D. hansenii,* gene expression profile of Δ*dhppz1* mutant was compared with parent strain by microarray. Out of total 6682 genes, 90 genes were annotated as up-regulated while 54 genes showed down–regulation of mRNA expression levels. 16.6% genes in up-regulated section were various

transporters, however different genes functioning in cytoskeleton organization, membrane trafficking, permeases, cell growth maintenance, mitochondrial membranes, cell wall and RNA processing also showed up-regulated expression. Some of up-regulated genes were still uncharacterized while some were unique to *D. hansenii*. Genes in down-regulated section were mainly transcriptional regulators. Other genes under this section were involved in salt stress pathway, nuclear proteins, transporters, cell growth maintenance, golgi membrane trafficking, mitochondrial membranes and cell wall with some uncharacterized and unique one.