A conserved Ser/Arg rich motif in PPZ orthologs from fungi is important for its role in cation tolerance

Anupriya Minhas#, Anupam Sharma#, Harsimran Kaur, Yashpal, Kaliannan Ganesan and Alok K Mondal*

Institute of Microbial Technology, Council of Scientific and Industrial Research (CSIR), Sector 39A, Chandigarh – 160 036, India

Running title: Ser/Arg rich motif in PPZ orthologs

*Correspondence to: Alok K. Mondal, Institute of Microbial Technology, Sector 39A, Chandigarh – 160 036, India. Tel: +91 1726665234; Fax: +91 1722690585; e-mail: alok@imtech.res.in

# Contributed equally

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Background - Ppz1 orthologs - novel family of phosphatases - have unique N-terminal non-catalytic domain

Results – Ppz1 ortholog from halotolerant yeast Debaryomyces hansenii plays important role in salt tolerance, cell wall integrity and cell growth in through distinct mechanism.

Conclusion – Short serine arginine rich motif in non-catalytic domain is essential for its role in salt tolerance.

Significance – This motif is conserved among orthologs and functionally important.

SUMMARY

PPZ1 orthologs, novel members of PPP family of phosphatases, are found only in fungi. They regulate diverse physiological processes in fungi e.g. ion homeostasis, cell size, cell integrity etc. Although they are an important determinant of salt tolerance in fungi, their physiological role remained unexplored in any halotolerant species. In this context, we report here molecular and functional characterization of DhPPZ1 from Debaryomyces hansenii which is one of the most halotolerant and osmotolerant species of yeast. Our results showed that DhPPZ1 knock out strain displayed higher tolerance to toxic cations and unlike in Saccharomyces cerevisiae, Na+ ⁄ H+ antiporter appeared to have important role in this process. Besides salt tolerance DhPPZ1 also had role in cell wall integrity and growth in D. hansenii. We have also identified a short, serine arginine rich sequence motif in DhPpz1p which is essential for its role in salt tolerance but not in other physiological processes. Taken together these results underscore a distinct role of DhPpz1p in D. hansenii and illustrate an example how organisms utilize the same molecular tool box differently to garner adaptive fitness for their respective ecological niches.

INTRODUCTION

Phosphoprotein phosphatases (PPP) constitute an important family of phosphatases that regulate plethora of cellular processes in eukaryotes 1-4. Based on their primary sequences, they are further classified into few distinct groups such as protein phosphatase 1 (PP1), PP2A, PP2B (commonly known as calcineurin), PP4, PP5, PP6 and PP7. Within the conserved catalytic domain (~30kD), members of PPP family contain three characteristics sequence motifs (GDxHG, GDxVDRG and GNHE) 2,4. Besides these, some groups also exhibit conserved sequence motifs outside the catalytic domain e.g. Ca2+-calmodulin binding motif in calcineurin, TPR motif in PP5 etc. which are very important for the functionality of the respective members 2, 4, 5 . Compared to the number of serine ⁄ threonine kinases, eukaryotic genomes contain fewer phosphatases that control specific dephosphorylation of thousands of phospho-protein substrates. Based on the evidences gathered from few well studied members, it appeared that the combinatorial associations with multiple regulatory subunits and other interacting proteins conferred potentially distinct substrate specificity to the catalytic subunits 4,6,7. Thus, the details of these interactions are of paramount importance in deciphering the biological roles of these phosphatases. PPP enzymes are among the ancient phosphatases, with at least one PPP-like member in all prokaryotes and eukaryotes. Fungi, in general, contain several PPP family of
phosphatase. Although the corresponding orthologs for most of them could be found in higher eukaryotes, a few of them are quite unique and restricted to this kingdom of life only.

Ppz1p and Ppz2p are such type of novel members of PPP family of phosphatase, originally identified in S. cerevisiae. Within the catalytic domain, Ppz1p shared approx. 60% sequence identity to PP1 and approximately 40% identity to PP2A making them distinct from those of PP1. PPZ1 orthologs appeared to regulate diverse physiological processes in fungi e.g. ion homeostasis, cell growth, cell size and integrity. In S. cerevisiae, deletion of PPZ1 leads to hyper tolerance phenotype towards sodium or lithium cations, which is more intensified by additional deletion of PPZ2. Deletion of PPZ2 alone has no effect on salt tolerance. Recent evidences indicated that Ppz1p affects cellular ion homeostasis primarily through the negative regulation of Trk1p, the major K+ transport system and this has substantial effect on salt tolerance and intracellular pH. Ppz1p has been shown to regulate the expression of Na+ efflux pump ENA1. Functional overlap of Ppz1p with other members of PPP family has also been documented in S. cerevisiae. Sit4p, a PP2A phosphatase and Ppz1p play opposite role in regulating G1/S transition. Complex genetic interaction of Ppz1p with Glc7p (an essential PP1 phosphatase) as well as with calcineurin/Crz1 pathway have also been observed. Hal3p is one of the most important regulators of Ppz1p. Over expression of Hal3p increases salt tolerance by suppressing the activity of Ppz1p. Hal3p has been shown to act as negative regulator by virtue of it binding to the catalytic domain of Ppz1p. Interactions between Hal3 and Ppz1 are destabilized upon increase in the intracellular pH. It has been suggested that the Hal3-Ppz1 complex acts as a pH sensor within the cell. Besides Hal3p, physical interaction of Ppz1p with quite a few other proteins has also been demonstrated in S. cerevisiae. Some of them e.g. Trk1p appeared to be its substrate while others e.g. Ypi1p regulates phosphatase activity. However, the details of the interactions of Ppz1p with these proteins are not known. Ppz1p contains a large amino terminal non-catalytic extension which is a characteristic feature of this group of phosphatases. This region is rich in few amino acids e.g. serine and arginine residues and has been shown to be functionally important.

PPZ1 is an important determinant of salt tolerance in yeast. Most of our understanding about this molecule is based on studying orthologs from very few species. Compared to these species, the yeast D. hansenii displays extreme halotolerance. In the recent past D. hansenii has been considered as a model organism to understand halotolerance in yeast. In this study, we have identified and cloned a PPZ1 ortholog (DhPPZ1) from D. hansenii. Functional characterization revealed that DhPPZ1 played important role in salt tolerance, cell wall integrity as well as in growth of D. hansenii through distinct mechanism. Most importantly, we have identified, for the first time, a ten amino acid long serine/arginine rich motif in the N-terminal non-catalytic domain of DhPpz1p which was essential for its role in salt tolerance but not in other physiological processes. This motif was not only conserved among orthologs but also functionally important as revealed from mutational analysis of S. cerevisiae Ppz1p.

**MATERIALS AND METHODS**

**Strains, Media and growth conditions -** Yeast strains and plasmids used in this study are listed in Table 1. D. hansenii strains were grown in YPD or YNB media at 28°C. For dilution spotting, freshly grown cultures (12 hours at 28°C) were normalized to OD₆₀₀ 1.0. Serial dilutions were made in sterile water and 5 μl of each of the 10⁻¹ to 10⁻⁴ dilutions were spotted onto YPD or YNB plates with required supplements.

**Construction of recombinant plasmids carrying DhPPZ1 and its mutants** - Plasmids pAN5 was constructed to express DhPpz1p from its native promoter in D. hansenii. For this, fragments of sizes ~0.8 kb (corresponding to the upstream of the ATG codon) and 1.7 kb (corresponding to DhPPZ1 ORF) were amplified from genomic DNA of CBS767 using primer pairs DhPPZ1_F/DhPPZ1_int_R1 and DhPPZ1_int_F1/DhPPZ1_int_R1. The amplified fragments were digested with BamH1/Nco1 and Nco1/Sac1 respectively and cloned at BamH1-Sac1 site of plasmid pAG-CfARS to obtain pPA1. Subsequently, a ~1.9 kb fragment corresponding to DhARG1 gene was cloned at Cla1 digested and klenow treated pPA1 to obtain pAN5. To
construct pAN6, a 610 bp fragment corresponding to DhtEF promoter was PCR amplified from genomic DNA using the forward DhtEF_F and DhtEF_R primers. This fragment was digested with BamH1 and Nco1 and cloned at BamH1-Nco1 site in plasmid pAN5 replacing DhpPZ1 promoter.

pAN4 plasmids was constructed to express DhPpz1p-RFP fusion protein in D. hansenii. 2.5kb fragment (corresponding to DhpPZ1 ORF along with its native promoter) was amplified using the forward DhpPZ1_Full F and reverse DhpPZ1_ORF RFP R primers and cloned at SalI/Xho1 sites in plasmid pDH1130 to obtain pAN4.

For constructing different N-terminal mutants (pAN51, pAN52, pAN53, pAN54, pAN55 and pAN56) the PCR based overlap extension method was followed31. Two separate PCR reactions were performed by utilizing the primer pairs, forward DhpPZ1_int F1 with corresponding reverse mutagenic primer and corresponding forward mutagenic primer with reverse DhpPZ1_int R2 primers to obtain overlapping mutated products. These products were combined in third PCR reaction using primers DhpPZ1_int F1 and DhpPZ1_int R2. The amplified fragment (~950 bp) was digested with Nco1 and BsrG1 and cloned into Nco1 and BsrG1 digested DhpPZ1-F RFP R primers and cloned at SalI/Xho1 sites in plasmid pDH11 to obtain pAN4.

To make deletion in DhpMPK1, a 2.2 kb DNA fragment harboring DhpMPK1 was PCR amplified using the forward primer DhpMPK1_F and reverse primer DhpMPK1_R using genomic DNA from D. hansenii strain CBS767 as template. The PCR fragment was cloned at BamHI/XhoI sites in pGEM7Z vector to obtain plasmid pAN7. Subsequently, the disruption cassette was constructed by replacing a 351bp EcoR1/EcoRV fragment of DhpMPK1 ORF with 3.2-kb DhhIS4 gene. The resultant plasmid (pAN7-HIS4) was then digested with BamHI/XhoI to isolate a ~5.2-kb disruption cassette. This cassette was transformed into DBH93 and the transformants obtained on YNB plates supplemented with arginine, were analyzed by PCR to confirm deletion in DhpMPK1. One of the transformants DBH932 was used subsequently.

**Determination of intracellular Li\(^{+}\) and K\(^{+}\):** To determine the intracellular concentration of Li\(^{+}\) and K\(^{+}\) in D. hansenii, the method described by Posas et al12 was essentially followed. Briefly, the cells from 100 ml culture at early logarithmic phase (OD\(_{600}\) ~1.0) were harvested by centrifugation, quickly washed twice with chilled milliQ water and finally re-suspended in 25 ml of incubation buffer (10 mM Tris-HCl, 50 mM MgCl\(_2\)) supplemented with or without 200mM LiCl salt. Cells (4 ml) were collected at different time intervals (0, 60, 90 and 120 min) on 0.45µm membrane filters. The filters were washed twice with chilled buffer (20 mM MgCl\(_2\), 1M sorbitol). Cation was extracted from the filters by incubating them in 10 ml extraction buffer (10mM Tris-HCl, 50 mM MgCl\(_2\)) supplemented with or without 200mM LiCl salt. (Shimadzu, Japan). Steady state concentration of cation (Li\(^{+}\) and K\(^{+}\)) was expressed as ppm/OD\(_{600}\) of cells.

**Quantitative Real-Time PCR assay:** The RT-qPCR was performed with the SYBR® green
one-step RT-qPCR Kit (Invitrogen). Each reaction was performed with 10 µl of 2X SYBR® Green Reaction Mix, 200 nM Gene specific-Forward primer, 400 nM Gene specific-Reverse primer (Supplemental Table 1), 100 nM Floressence (reference dye), 0.8 U SuperScript™ III RT/Platinum® Taq Mix and 500-1000 ng RNA template in 20µl total reaction volume. The PCR reaction was carried out in i-cycler iQ™ (BioRad). Reaction conditions for one step RT-PCR includes initial cDNA synthesis at 55°C for 45 min 15 sec, followed by initial denaturation at 95°C for 2 min, and 40 cycles at 95°C for 15 sec, 55°C for 30 sec and 72°C for 20 sec. Amplification specificity was assessed using melting curve analysis. Relative expression levels were normalized using DhGPDH as an internal control. The average relative expression levels for each gene in wild type and dhppz1 mutant was calculated using the ΔΔCt method and expressed as fold difference. The RNA was made free of DNA contamination by in-column DNase-I (Invitrogen) digestion. The absence of the DNA contamination in the RNA samples was confirmed by a reaction with platinum Taq DNA polymerase without reverse transcriptase.

Two-hybrid assay - The two-hybrid assay was carried out as described earlier 32. Plasmids pEG202 and pJG4-5 were used for constructing the bait and prey respectively 33. DhHAL3 ORF (1.77 Kb) was PCR amplified using primers DhHAL3 ORFf and DhHAL3 ORFr and cloned in pJG4-5 at EcoR1 (blunt-ended) and Xho1 site. DhPPZ1 and DhPPZ1Δ27-36 was cloned in bait vector pEG202 at BamH1-Xho1 site. S. cerevisiae strain EGY 48 was co-transformed with bait and prey constructs and selected on minimal media with 2% glucose and without tryptophan and histidine. Co-transformed cells were grown up to OD600 ~1.0 and 5µl of the cultures (normalized to OD600 1.0) were spotted onto minimal media with 2% galactose, 1% raffinose and without tryptophan, histidine and leucine. Plates were incubated for four days at 30°C. For liquid growth assays, EGY48 carrying DhHAL3 as prey and DhPPZ1 or its mutant as bait were grown overnight in minimal media with 2% raffinose (without tryptophan and histidine). The cultures were re-inoculated in minimal media with 1% raffinose and 2% galactose (without tryptophan, histidine and leucine) at an initial OD600 of ~0.10 and grown for 41 hrs. Experiments were carried out twice in duplicate with pool of four independent transformants.

RESULTS

Identification and Cloning of PPZ1 ortholog from D. hansenii - To identify the gene encoding PPZ1 ortholog in D. hansenii, a TBLASTN search (www. ncbi.nlm.nih.gov) was carried out using protein sequences of S. cerevisiae Ppz1p (692 amino acids) and Ppz2p (710 amino acids) as query against the available genome sequence database of D. hansenii strain CBS767. A single 572 amino acid long hypothetical protein was identified as putative PPZ1 phosphatase in D. hansenii. DhPPZ1 ORF sequence was found to be located at chromosome E and its length was 1719bp. Predicated amino acid sequence of DhPpz1p showed 54.8% and 54.2% identity with Ppz1p and Ppz2p respectively. Further, like a typical Ppz1p ortholog, DhPpz1p also contains a large, N-terminal non-catalytic region consisting of 254 amino acids and C-terminal 318 amino acids corresponding to the catalytic domain. C-terminal catalytic region of DhPpz1p showed 85.2% and 83.4% identity with that of Ppz1p and Ppz2p respectively (Supplemental Fig. S1). In the N-terminal half, DhPpz1p exhibited only 25.0% and 35.4% identity with that of Ppz1p and Ppz2p of S. cerevisiae respectively. However, like Ppz1p and Ppz2p, the N-terminal non-catalytic region of DhPpz1 is also rich in serine (63 residues) and asparagine (45 residues).

Ppz1p is a membrane protein in S. cerevisiae and its membrane localization is due to the myristoylation at Gly 2 residue 14. This residue is appeared to be conserved in DhPpz1p. Therefore, intracellular localization of DhPpz1p was determined. For this purpose, the plasmid pAN4 was made to express DhPpz1p as RFP fusion protein and transformed into D. hansenii strain DBH9. The transformants were grown to logarithmic phase and examined by fluorescence microscopy. In the cells expressing the DhPpz1p-RFP fusion protein, RFP fluorescence was visible throughout the cytoplasm which indicated that unlike Ppz1p, DhPpz1p in D. hansenii was localized in the cytosol (Supplemental Fig. S2).

DhPPZ1 knock-out strain shows increase tolerance to toxic cations - PPZ1 homolog has been shown to be an important determinant of salt tolerance in S. cerevisiae and the deletion of this gene resulted in the increased tolerance to salt in this species 12. We have identified only one PPZ1 homolog in D. hansenii. To determine
the role of this gene in salt tolerance, *D. hansenii* strain (DBH91) carrying deletion in *DhPPZ1* gene was created and the growth pattern of this strain were checked on plates containing different concentration of LiCl and NaCl. Compared to the control strain DBH9 which did not grow beyond 100 mM of LiCl, DBH91 showed prominent growth even at 1M LiCl. However, on plates containing 1M or 2M NaCl, no perceptible difference in growth was observed between these two strains (Fig 1A).

Effect of *DhPPZ1* mutation on salt tolerance was also measured in liquid medium. For this, DBH9 and DBH91 strains were grown in YPD media or YPD media supplemented with different concentrations of salt for 24h. Relative growth of the strain at each concentration of salt was expressed as percentage of the growth (OD$_{600}$) in YPD medium without salt at respective pH was used as control (100%). It was apparent from the results that DBH91 grew better than DBH9 at all concentrations of LiCl tested. In the presence of NaCl, DBH91 exhibited 4-6 times more growth at 0.5 and 1.0 M NaCl but at higher concentration the difference was negligible (data not shown). Thus the deletion of *DhPPZ1* also conferred higher salt tolerance in *D. hansenii*. Next we checked whether pH of the growth medium has any effect on this increased resistance to salt stress. For this, DBH9 and DBH91 strains were re-inoculated in 25ml YPD medium containing indicated concentrations of NaCl with pH adjusted to 6.0, 7.0 and 7.5. After 24h of incubation at 28°C, OD$_{600}$ of these cultures was measured and the data was expressed as their relative growth. Growth (OD$_{600}$) in YPD medium without salt at respective pH was used as control (100%). It was observed that, when pH of the medium was shifted to higher values from pH6, the favorable effect of 0.5M NaCl on the growth of the mutant strain started decreasing and it almost diminished at pH 7.5 (Fig 1B). Similar effect of pH on sodium tolerance was also observed at higher salt concentrations (Fig 1B).

Besides alkali metal cations, *ppz1* mutant in *S. cerevisiae* strain can also tolerate high concentration of other toxic cations such as spermine, tetramethyl ammonium and hygromycin B. To check whether *D. hansenii* mutant also exhibit similar phenotype, growth of DBH91 and DBH9 stain on YPD plates supplemented with different concentrations of hygromycin and spermine were checked by dilution spotting. Our results showed that DBH91 strain could grow in the presence of 50µg hygromycin or 4.5M spermine. In contrast, DBH9 strain failed to grow above 10 µg hygromycin or 2.5M spermine containing plates (Fig 1C). The *dhppz1* mutant DBH936 also showed similar phenotype.

*DhPPZ1* knock-out strain accumulate lower amount of Li$^+$ intracellularly - As mentioned above, *dhppz1* mutant strain exhibited higher tolerance to toxic cation. This phenotype could arise if it accumulates less amount of toxic ion in comparison to parent strain. To examine this possibility, steady state concentration of Li$^+$ ions inside the cells (Li$^+$$^{\text{int}}$) was determined. *dhppz1* mutant (DBH936) cells at early logarithmic phase, was exposed to 200mM LiCl at indicated pH. Samples were withdrawn at different time interval and the total intracellular Li$^+$ ion content of the cells was measured by atomic absorption spectrophotometer as described in Materials and Methods. From our results it was apparent that *dhppz1* mutant strain accumulated lower steady state concentration of lithium ion compared to the parent strain both at pH 6.0 and at pH 7.0 (Fig 2A).

In *S. cerevisiae*, Ppz1p modulates the activity of Trk K$^+$ transporter. Increase in the activity of Trk1p in the absence of Ppz1p results in the accumulation of higher K$^+$ ion concentration inside the cell which indirectly affects the salt tolerance. The increased resistance to LiCl exhibited by *dhppz1* mutants could also be due to higher accumulation of K$^+$ intracellularly. To check this, intracellular level of K$^+$ ion was determined in *dhppz1* mutant strain (described in Materials and Methods). DBH936 as well as parent DBH93 cells at early logarithmic phase were exposed to 200mM LiCl at different pH. The cells were harvested by vacuum filtration at different time points and the intracellular K$^+$ concentration in these cells was measured by atomic absorption spectrophotometer. Results presented in Fig 2B showed that *dhppz1* strain accumulated higher K$^+$ compared to the parent strain both at pH 6 and pH 7.

Activity of various transporters directly regulates the intracellular concentration of different cations. The above mentioned difference in Li$^+$ and K$^+$ observed in *dhppz1* mutant could be a result of differences in the level of cation transporters. It is to be noted that the loss of Ppz1p activity induces *ENA1* transcription in *S. cerevisiae*. Therefore the level of expression of major cation transporters *DhENA1*, *DhTRK1* and *DhNHA1* were compared in *dhppz1* mutant.
and parent strain using Real time PCR method. Under the salt stress, dhppz1 mutant showed 10 fold higher DhNHA1 expression compared to the parent strain used as wild type control (Fig 2E). In comparison, little or no difference in the level of expression of DhENA1 and DhTRK1 could be observed between dhppz1 mutant and parent strain under these conditions (Fig 2C and 2D). DhPPZ1 is crucial for normal cell growth in D. hansenii - PPZ1 plays regulatory role in number of divergent pathways such as salt stress, cell wall remodeling and cell cycle regulation in yeast. Disruption of DhPPZ1 showed that it was not an essential gene in D. hansenii, however it did not rule out its vital role in normal cellular processes. To check this, growth pattern of the dhppz1 strain was compared with the parent strain. Saturated cultures of D. hansenii strains (DBH936 and DBH93) were re-inoculated into 100 ml YPD medium at initial OD600 of 0.05 and incubated further at 28°C with vigorous shaking (200 rpm). Growth of the cultures was monitored by measuring OD600 at regular interval. It was evident that dhppz1 mutant (DBH936) grew significantly slower than the parent strain DBH93 used as control (Fig 3A). The growth defect exhibited by dhppz1 mutant of D. hansenii could be abrogated by expressing DhPPZ1 either under its own promoter (pAN5) or a stronger DhTEF promoter (Fig 3B). Moreover, the growth of the strain expressing DhPPZ1 under DhTEF promoter was quite similar to DBH93 thereby clearly indicating that the over expression of DhPPZ1 had negligible effect on the growth of D. hansenii. Contrasting this, an excess of Ppz1p S. cerevisiae is known to have adverse effect on growth by affecting G1/S transition, bud emergence and DNA synthesis 9, 11, 16. Although S. cerevisiae strains lacking both PPZ1/2 show normal growth in YPD medium, however with the increase in the pH of the medium it exhibits a slow-growth phenotype 11. Therefore the growth of the dhppz1 mutant strain was checked in YPD media at pH 6.0, 7.0 and 7.5. As shown in Fig 3C, the growth pattern of dhppz1 mutant was quite similar at pH 6.0, 7.0 and 7.5 which indicated that the deletion of DhPPZ1 in D. hansenii had no significant effect on growth under alkaline condition.

Effect of DhPPZ1 mutation on cell wall remodeling in D. hansenii - To check the involvement of DhPPZ1 in cell wall remodeling in D. hansenii, the sensitivity of dhppz1 mutant towards cell wall destabilizing agents, such as caffeine was determined by dilution spotting. The strain DBH93 which was used as control grew very well on plates containing 5 mM caffeine. In contrast, DBH936 did not grow at all on this plate. However, with the addition of 1 M sorbitol as osmotic stabilizer, the growth of DBH936 in the presence of 5 mM caffeine could be seen (Supplemental Fig S3A). Mutation in dhppz1 gene also conferred temperature sensitive phenotype in D. hansenii as DBH936 failed to grow at YPD plates incubated at a temperature of 32°C and above. This defect could also be alleviated by the addition of 1 M sorbitol as osmotic stabilizer (Supplemental Fig S3B). These results clearly indicated important role of DhPPZ1 in cell wall integrity in D. hansenii as well.

In yeast, a signal transduction pathway comprising of MAP kinase MPK1 plays pivotal role in mediating cell wall remodeling under different physiological conditions including stress 35. Genetic interaction of this pathway with PPZ1 has been reported in S. cerevisiae earlier 11, 34. Through BLAST analysis of the genome sequence database we had identified a putative MPK1 ortholog (DhMPK1) in D. hansenii. To determine the genetic interaction between this pathway and DhPPZ1 we have constructed DBH932 strain carrying deletion in DhMPK1 gene. DBH932 displayed temperature-dependent cell lysis defect which could be abrogated by addition of 1 M sorbitol (Supplemental Fig S4). These phenotypes suggested that DhMPK1 was a typical member of the cell wall integrity MAPK pathway in D. hansenii. We next transformed DBH932 strain with pAN5 (DhPPZ1 gene under its native promoter) and pAN6 (DhPPZ1 gene under strong DhTEF promoter) and transformants were selected on YNB plates. Phenotype analysis of the transformants showed that only the expression of DhPpz1p from a strong promoter but not from its own promoter could suppress caffeine and salt sensitivity of DBH932 (Fig 4A). Similarly, the growth defect of dhmpk1 deletion could also be abrogated only by over expressing DhPpz1p (Fig 4B). Thus, like that in S. cerevisiae, DhPPZ1 behaved like a multi-copy suppressor of the growth defect associated with the deletion of the cell wall integrity pathway component 11. N-terminal non-catalytic region is essential for the functionality of DhPpz1p - Ppz1p represents a novel phosphatase family with a conserved, catalytic C-terminal region and a large non-catalytic N-terminal region rich in few amino
acids e.g. serine, asparagine, arginine residues \(^8\). The C-terminal catalytic domain shares substantial sequence homology among Ppz1p orthologs as well with other PPP members. In comparison, the N-terminal regions are quite diverged in their primary sequences. The N-terminal region is a unique feature of Ppz1p orthologs and appeared to have regulatory role in the functionality of this phosphatase. Surprisingly, not much information is available about this region. Analysis of DhPpz1p sequence showed that the N-terminal region comprised of amino acid residues 1-278 and the catalytic domain was from amino acid residue 279-572. Phenotypic analysis showed that either N-terminal (pAN9) or C-terminal catalytic (pAN10) region was non functional as they failed to suppress LiCl and hygromycin tolerance or caffeine sensitive phenotype of \(dhppz1\) mutant (data not shown). Besides these, none of these constructs could rescue the slow growth phenotype exhibited by \(dhppz1\) mutant (data not shown). Together these results validated the importance of both the halves for the functionality of DhPpz1p. Primary sequence clearly indicated that N-terminal of DhPpz1p was also rich in serine and asparagine residues as it contained sixty three serine (22.58%) and forty five asparagine (16.13%) residues. Analysis of the primary sequence of N-terminal region (1-278 aa) of DhPpz1p with the help of bioinformatics tools such as SMART (Simple Modular Architecture Research Tool at EMBL) and PROSITE (database of protein families and domains) did not indicate the presence of any known domain or motif in this region. However, it showed six sequence motif of low complexity that are rich in serine, asparagin or arginine residues (Fig 5). To delineate the role of these regions in the functionality of DhPpz1p, six mutant constructs were generated by PCR based method by deleting the amino acid residues corresponding to 27-36 (pAN51), 59-75 (pAN52), 81-104 (pAN53), 106-120 (pAN54), 162-183 (pAN55) and 241-254 (pAN56). All these constructs were transformed in \(D. hansenii\) strain DBH936. Transformants were selected on YNB plates and were assessed for their ability to complement phenotype associated with \(dhppz1\) mutation. For this, serial dilutions of log phase cultures of DBH936 cells harboring pAN51, pAN52, pAN53, pAN54, pAN55 and pAN56 plasmid were spotted on YNB plates, supplemented with different concentrations of LiCl, hygromycin or caffeine. Growth on plates was observed after 3-4 days of incubation at 28\(^{\circ}\)C (Fig 5). DBH936 strains transformed with plasmid pAN51 and pAN54 remained LiCl resistant similar to DBH936 (\(dhppz1\)) strain, while all other mutant constructs fully reverted to lithium sensitive phenotype similar to DBH936 expressing wild type DhPpz1p. On hygromycin plate, DBH936 transformed with only pAN51 showed hygromycin resistant phenotype similar to that of \(dhppz1\) mutant whereas with all other constructs hygromycin sensitive phenotype was observed. These results thus indicated that pAN51 encode a non-functional allele of DhPpz1p with respect to this phenotype. \(D. hansenii\) strain DBH936 was highly sensitive to caffeine and therefore it did not grow on plate containing caffeine. In contrast, DBH936 strain transformed with all the mutant constructs were able to grow on these plates which clearly indicated that all the mutant constructs behaved like wild type DhPpz1p in this regard (Fig 5).

Next we determined whether these mutant constructs could rescue the slow growth phenotype of \(dhppz1\) mutant observed earlier. For this, saturated cultures of DBH936 strain transformed with all these constructs were re-inoculated into 50 ml YNB medium at initial \(O.D_{600}\) of 0.025. After 24h of growth at 28\(^{\circ}\)C, \(O.D_{600}\) of each culture was measured and the data was expressed as their relative growth compared to the parent DBH93 strain used as control. Our results revealed that all mutant constructs could suppress the growth defect of \(dhppz1\) strain indicating their functional activity in vivo. Interestingly, DBH936 harboring pAN51 exhibited two fold more growth than the parent strain DBH93 used as controls in this experiment (Supplemental Fig S5).

A conserve serine/arginine rich motif in N-terminal non-catalytic region is important for cation tolerance - From the above analysis, it thus appeared that none of the motifs was important for DhPpz1p towards its role in cell wall remodeling or cell growth. However, the short sequence motif corresponding to 27-36 amino acid residues was very important for both lithium and hygromycin tolerance phenotype and appeared to be indispensable for DhPpz1p towards its role in ion homeostasis. This motif was rich in serine and arginine residues and it contained five serine and three arginine residues. Analysis of the sequence of few other orthologs from different yeast and fungal species clearly showed the presence of this motif in them and
thus it appeared to be evolutionarily conserved among the members of this group (Fig 6A). The sequence alignment of this motif showed that the residues Ser27, Ser30, Ser36, Arg29 and Arg32 were present in all however Ser33 appeared to be conserved only in yeast and not in filamentous fungi. Interestingly, the 8th and 9th positions were always occupied by an arginine along with a bulky hydrophobic residue. To confirm the functional importance of the conserved residues we have created few mutant constructs by site directed mutagenesis. In these mutants, serine residues were changed to alanine whereas the arginine residues were changed to either alanine or glutamic acid. The plasmids carrying these mutants were transformed in D. hansenii strain DBH936. The growth patterns of the transformants were assessed by dilution spotting on YNB plates containing different concentrations of LiCl or hygromycin. The result of this experiment was summarized in Fig 6B. Mutation of conserved serine and arginine residues together (SRAA or SRAE) could not suppress lithium or hygromycin resistant phenotype of the dhppz1 mutant. The mutation of only serine or arginine residues alone (4SA, 3RE or 3RA) also could not suppress this phenotype. Replacement of arginine residues with either alanine or glutamic acid (3RA or 3RE) had similar effect. These results clearly indicated that both the serine and arginine residues have crucial roles in the functionality of DhPpz1p. This motif is a common feature among Ppz1p orthologs (Fig 6A). To check the functional importance of this motif in other orthologs, we constructed a mutant of S. cerevisiae PPZ1 (PPZ1-∆43-52) that will express a Ppz1p allele devoid of this motif. To this effect, we undertook molecular characterization of PPZ1 ortholog from D. hansenii which is one of the most halotolerant species of yeast and considered as model organism to understand halotolerance in yeast 27,29. Interestingly, this report is also the first documentation of functional characterization of any D. hansenii gene in its native host.

Analysis of sequence database identified only one PPZ1 ortholog among the eight members of PPP family that are present in D.
ortholog of each group that are present in *S. cerevisiae* contains twelve such proteins. Except for the *PPH3* ortholog, *D. hansenii* contains at least one ortholog of each group that are present in *S. cerevisiae* and redundant or partially redundant isoforms are notably absent in this species (our unpublished observation). From the primary sequence, DhPpz1p appeared to have all the features typical to this type of proteins. To determine its role in *D. hansenii*, phenotypic analysis of *DhPPZ1* knock-out strains (DBH91 and DBH936) was carried out. *dhppz1* mutant exhibited high level of resistance towards different toxic cations such as Li\(^+\), hygromycin and spermine (Fig 1). For NaCl, better growth of the *dhppz1* mutant was apparent only at lower concentration and no significant difference in the growth was observed at 2.0 M or higher concentration of NaCl (Fig 1B). *dhppz1* accumulated less amount of Li\(^+\) compared to the parent strain both at pH6.0 and 7.0. Lower steady state concentration of toxic cation in the cell was expected to lead to the higher resistance towards these ions. Besides this, the intracellular K\(^+\) concentration appeared to be elevated in the mutant (Fig 2). Higher K\(^+\) in the cell could also affect the accumulation of toxic cation. In case of *S. cerevisiae* *ppz1* mutant, lower intracellular Li\(^+\) and increased level of intracellular K\(^+\) was also observed. This was achieved through two distinct mechanisms. One is through the transcriptional up-regulation of *ENA1* encoding Na-H\(^+\) ATPase – the major Na-efflux pump in *S. cerevisiae*. As a substrate, the major K\(^+\) transporter Trk1p is also regulated by Ppz1p. In the absence of Ppz1p, the increase in the activity of Trk1p resulted in the accumulation of K\(^+\) and thereby affecting the membrane potential and accumulation of toxic cation in the cell. In *S. cerevisiae*, interaction of Ppz1p with Trk1p has been demonstrated through immunoprecipitation 14. Both Ppz1p and Trk1p have been shown to be associated with the membrane as observed by GFP fusion as well as other methods and their physical proximity in the cell has been suggested to be crucial for Ppz1p to utilize Trk1p as substrate. Our results showed that DhPpz1p-RFP fusion protein was present in the cytosol and therefore the scenario was quite different in *D. hansenii*. Whether DhPpz1p could still modulate the activity of K\(^+\) transporter DhTrk1p in *D. hansenii* thus remains an open question.

We had observed that the mutation in *DhPPZ1* did not significantly affect the level of *DhENA1* transcript in *D. hansenii* which indicated the role of Ena1 independent mechanism in the salt tolerance of *D. hansenii* *dhppz1* mutant. Earlier studies showed that the extent of salt tolerance displayed by *S. cerevisiae* *ppz1* mutant remained unaffected by raising the pH of the medium to 8.5 12. Contrasting this, the salt tolerance of *D. hansenii* mutant diminished at higher pH (Fig 1B). This observation indicated a possible role of Na\(^+\)/H\(^+\) anti-porter DhNha1p. Our observation that under salt stress *DhNHA1* expression is strongly up regulated in *dhppz1* mutant further supports this view (Fig 2E). In yeast, the role of Nha1p orthologs in the regulation of cell cycle, cell volume, membrane potential and ion homeostasis has been demonstrated. Though both *S. cerevisiae* and *D. hansenii* accumulate less toxic cation in the absence of PPZ1, however this could be achieved through distinct mechanisms.

*DhPPZ1* is not an essential gene in *D. hansenii*. However, its deletion caused dramatic decrease in the growth of the mutant indicating its vital role in the cellular physiology in this species. Earlier studies showed that an excess of Ppz1p confer slow growth phenotype in *S. cerevisiae* by affecting G1/S transition and bud emergence 9,11, 16. In contrast, the over expression of *DhPPZ1* had negligible effect on the growth of *D. hansenii*. In *S. cerevisiae*, strains lacking PPZ1/2 showed pH dependent growth - a relatively slow-growth phenotype was observed on medium with increasing pH 13. Our result showed no significant changes in the growth of *dhppz1* mutant with the increase in the pH of the medium (Fig 3C). DhPpz1p appeared to have functional link with the cell wall integrity pathway in *D. hansenii*. Like a mutant having defect in cell wall integrity pathway, *dhppz1* strains also showed cell lysis defect at higher temperature and sensitivity toward cell wall destabilizing agents, such as caffeine. Both these defects could be suppressed by addition of 1M sorbitol to the medium (Supplemental Fig S3). In *S. cerevisiae*, PPZ1 act as multi copy suppressors of the defect associated with the mutation in *MPK1* 11. Similar phenotype was also observed with *DhPPZ1* in *D. hansenii*. Taken together these results underscore a distinct role of DhPpz1p in *D. hansenii* despite having some functional overlaps with its ortholog in *S. cerevisiae*. Both these species live in different ecological niche. Thus it is not strange that they have evolved with different
survival strategies using the same molecular elements but in distinct ways.

Like a typical member of its group, DhPpz1p possesses a large N-terminal non-catalytic region that is rich in few amino acid residues such as serine. Earlier studies with Ppz1p showed that it is essential for the functionality. Deletion of this region in DhPpz1p also rendered the molecule non-functional. In order to have structure-function insight about this region, we undertook bioinformatics analysis which did not show the presence of any known domain or motif but identified six regions of short sequence motif with low complexity. To check the role of these motifs, deletion constructs (pAN51, pAN52, pAN53, pAN54, pAN55 and pAN56) were created and subjected to phenotype analysis (Fig 5 and supplemental Fig S5). All the mutants could complement the caffeine sensitivity and growth defect of *dhppz1* mutation similar to that of wild type DhPpz1p. Compared to other constructs, copious growth was observed with pAN51. Therefore, all these motifs were dispensable for the role of DhPpz1p in cell integrity and cell growth. Surprisingly, pAN51 failed to suppress the lithium and hygromycin resistance indicating the motif 27-36 could be indispensable for salt tolerance. The growth of pAN54 on LiCl but not on hygromycin containing plate indicated a subtle, yet unexplained, difference in these two as toxic cation. Nevertheless, analysis of these constructs clearly demonstrated that the participation of DhPpz1p in salt tolerance, cell integrity pathway or cell growth occurred through distinct molecular events. The sequence motif 27-36 was rich in serine and arginine residues that are indispensable for its function in salt tolerance. The discovery that this motif is highly conserved among the Ppz1 orthologs also suggests its importance. Mutational analysis of *S. cerevisiae* Ppz1p further supports this view (Fig 6). Previous studies suggested that Hal3p binds to the catalytic moiety of Ppz1p in *S. cerevisiae* and the N-terminal non-catalytic half of Ppz1p might play a protective function against the inhibition by Hal3p\(^{18,20}\). The Ser/Arg rich motif identified in this study is present in the N-terminal region of DhPpz1p which is far outside of the catalytic domain and therefore likely to have, if any, a built in switch like function in preventing the interaction with DhHal3p. However, the yeast two hybrid assay clearly showed that this motif had no role in binding DhHal3p (Fig 7). Recently serine/arginine rich motifs have been shown to be involved in protein-protein interaction\(^{36,37}\). It is unclear at present how this motif could be functioning. As a member of PPP family of phosphatases, the spectrum of activities exhibited by DhPpz1p is regulated by its interactions with different cellular proteins. It is plausible that some of these interactions are mediated by the serine-arginine rich motif.
Acknowledgement:
A.M, A.S and H.K are the recipients of Senior Research Fellowships from the Council of Scientific and Industrial Research, India. We are thankful to R. Sharma and D. Bhatt for excellent technical assistance and Ms A. Sukla for atomic absorption spectroscopy.

REFERENCE


### TABLE1. Strains and plasmids used in this study

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Figure Legends:

Figure 1. Growth of dhppz1 mutant in the presence of toxic cations.
(A) 5μl of ten-fold serial dilutions of logarithmic phase culture of D. hansenii strains DBH9 (wild type) and DBH91 (dhppz1) were spotted on YPD plates supplemented with different concentrations of LiCl and NaCl. Growth on plates was observed after 2 days incubation at 28ºC.

(B) Saturated cultures of DBH9 and DBH91 grown in YPD medium were re-inoculated (at initial OD_{600} of 0.025) into 25ml YPD medium at different pH containing different concentrations of NaCl. After 24h of growth at 28ºC, the OD_{600} of these cultures was measured. Relative growth of the culture at each concentration of salt was expressed as percentage of the growth measured in the absence of added salt at same pH. Data presented is results of three independent experiments (mean ± sd).

(C) Dilution spotting of DBH9 and DBH91 on YPD plate containing different amount of hygromycin and spermine. Results are representative of three independent experiments.

Figure 2. Effect of DhPPZ1 knockout on cation ion transport in D. hansenii.
(A & B) Intracellular concentration of Li^+ and K^+ in wild type D. hansenii (DBH 93) and dhppz1 mutant (DBH 936) at different pH. D. hansenii cells at early log phase were exposed to 200mM LiCl in incubation buffer at different pH. Cells were collected at different time points by filtration and the total intra cellular concentration (ppm per OD_{600} of the cells) of Li^+ and K^+ ion in these cells were measured by atomic absorption spectrophotometer as described in “Materials and Methods”. Data (mean ± sd) from duplicate biological samples with three replicates each are shown.

(C-E) Comparison of the level of DhENA1, DhTRK1 and DhNHA1 transcript in wild type and dhppz1 strains as analyzed by RT-qPCR. Total RNA was isolated from logarithmic phase culture of wild type (DBH9) and dhppz1 mutant (DBH91) strain grown in YPD medium or after exposing them to 200 mM LiCl for 20 min. The RT-qPCR was performed with one step SYBR-I green reaction kit (Invitrogen) using gene specific primers. Relative expression levels were normalized using DhGPDH as an internal control. The average relative expression levels for each gene in wild type and dhppz1 mutant was calculated using the ΔΔCt method and expressed as fold difference (mean ± sd).

Figure 3. DhPPZ1 knock out affects growth of D. hansenii.
(A) Growth curve of dhppz1 mutant (DBH936) and control parental (DBH93) D. hansenii strains are shown. Saturated cultures DBH936 and DBH93 were reinoculated 100 ml YPD medium at initial OD_{600} of 0.05. Growth of these cultures was monitored by measuring OD_{600} at regular intervals.

(B) Reversal of slow growth phenotype of dhppz1 mutant by expressing DhPPZ1 from plasmid. Saturated cultures of DBH936 strains transformed with indicated plasmids were reinoculated into 25ml YPD medium (at initial OD_{600} of 0.025). After 24h of growth at 28ºC, OD_{600} of these cultures was measured. DBH93 strain transformed with plasmids pDA1 and pDH4 was used as control. Data was expressed as their relative growth with respect to the control.

(C) Relative growth of dhppz1 mutant at different pH. Saturated cultures of DBH936 and DBH93 (control) were reinoculated (initial OD_{600} 0.025) into 50 ml YPD medium buffered to different pH. After 24 h of growth at 28C OD_{600} of the culture was measured. The growth at pH 6.0 for the respective culture was taken 100%. Data presented is the results of three independent experiments (mean ± sd).

Figure 4. Over-expression of DhPpz1p suppresses phenotypic defects of dhmpk1 deletion in D. hansenii.
(A) Suppression of the toxic cation and caffeine sensitivity of dhmpk1 mutant. DBH932 harboring plasmids pAN5, pAN6 or pDA1 (vector control) were grown in YNB medium to logarithmic phase (OD_{600} ~ 1.0). Ten fold serial dilutions of these cultures were on YNB plate containing LiCl or caffeine as indicated.

(B) Relative growth of dhmpk1 mutant harboring different plasmids as indicated. OD_{600} after 24h of growth for control (DBH93 harboring pDA1 and pDH4) was taken 100%. Data presented is results of three independent experiments (mean ± sd).
Figure 5. Structure-function analysis of N-terminal domain of DhPpz1p. Cartoons depicting wild type and mutant DhPpz1p. C-terminal catalytic domain (light box), N-terminal non-catalytic domain (dark box) and the position of different deletions are shown. Right panel shows phenotypic complementation of *dhppz1* mutation in DBH936 by different plasmid constructs as determined by dilution spotting on YNB plates containing LiCl, hygromycin (Hyg) and caffeine (Caf). + indicate complementation and – indicate not complementation. Experiments were repeated three times with similar results.

Figure 6. Mutational analysis serine/arginine rich motif in DhPpz1p and Ppz1p.
(A) Sequence alignment of serine/arginine rich motif present in different Ppz1p orthologs from different species YL (*Yarrowia lipolytica*: XP_504610.2) CD (*Candida dubliniensis*: XP_002422157.1), Pz11 (*Neurospora crassa*: AF071752), CL (*Clavispora lusitaniae*: XP_002619471), PG (*Pichia guilliermondii*: EDK41476.2), Ppz1 (*S. cerevisiae*: NP_013696.1) Ppz2 (*S. cerevisiae*: NP_010724.1) and DhPpz1 (*D. hansenii*: XP_459586.2). Conserved residues are shown in asterisk.
(B) Phenotypic complementation of wild type DhPpz1p and mutants carrying point mutation in serine/arginine rich motif along with the sequence of respective mutated region are shown. + indicate complementation and – indicate not complementation.
(C) Dilution spotting of *S. cerevisiae* strains expressing wild type Ppz1p or its mutants on YNB plate containing LiCl is shown. PPZ1 (parental strain BY4742/pRS423), ppz1 mutant (Y10557/pRS423) and ∆43-52 (Y10557/PPZ1-∆43-52) were grown overnight on YNB without histidine before serial dilution. Representative data of three independent experiments are shown.
(D) Effect of mutation in individual serine and arginine residues of Ser/Arg motif on salt tolerance exhibited by dhppz1 mutant. Ten fold serial dilution of DBH936 strain expressing different point mutations was spotted on YNB plate containing 0.4 and 0.6 M LiCl. Plated were incubated at 28°C for 3-4 days before being photographed. Culture of DBH936 harboring empty vector pDA1 was used as control. Representative data of two independent experiments are shown.

Figure 7. Role of Ser/Arg rich motif in the interaction of DhPpz1p with DhHal3p
(A) Two hybrid assay for interactions between DhHal3p and DhPpz1p. Wild type DhPPZ1 or its mutant having deletion in Ser/Arg motif (∆27-36) cloned in pEG202 vector was used as bait whereas DhHAL3 cloned in plasmid pJG4-5 was used as prey. Bait and prey constructs (in pairs as indicated) or in combination with empty prey or bait (as control) were transformed into *S. cerevisiae* strain EGY48. Growth of the transformants on Gal-Raf minimal medium is shown after dilution spotting. Experiments were repeated twice with pool of four independent transformants. Representative data is shown.
(B) Growth of EGY48 harboring DhPPZ1 and ∆27-36 bait along with empty prey or DhHal3 in prey vector were grown overnight in minimal media with 2% raffinose (without tryptophan and histidine). The cultures were re-inoculated in minimal media with 1% raffinose and 2% galactose (without tryptophan, histidine and leucine) at OD₆₀₀ ~0.10 and grown further for 41 hrs. Data (mean ± sd) of two independent experiments each performed in duplicate with a pool of four different transformants is shown here.
Figure 1
Figure 2
Figure 3
Figure 4

A

LiCl

YNB 0.6M 0.8M

Caffeine

20mM 22mM

B

Relative growth (%)

pDA1 pAN5 pAN6 Control

Figure 4
Figure 5

Plasmid

- pAN51 (Δ27–36)
- pAN52 (Δ59–72)
- pAN53 (Δ81–104)
- pAN54 (Δ106–120)
- pAN55 (Δ162–183)
- pAN56 (Δ241–254)
- pAN5 (DhPPZ1)

Complementation

- Ltr
- Hyg
- Cmr

- + + +
- + + +
- + + +
- - + +
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- + + +
- + + +
Figure 6

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Figure 7
A conserved Ser/Arg rich motif in PPZ orthologs from fungi is important for its role in cation tolerance
Anupriya Minhas, Anupam Sharma, Harsimran Kaur, Yashpal FNU, Kaliannan Ganesan and Alok K. Mondal

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