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"Role Of Potential G-Quadruplex Forming Sequences In The Salt Tolerance Properties Of *Saccharomyces Cerevisiae* "

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ABSTRACT:
DNA is riveting on account of its information carrying capacity, generation after other, but one of its phenomenal character is the formation of a non-canonical structure due to its highly polymorphic nature, called G- quadruplexes, organized as by GnX1–7 GnX1– 7GnX1–7Gn residues. As the promoter region of the genes contains G4 runs, it indicates their possible functions in transcriptional regulation of genes. A study was done to find a verdict on the ability of PG4 motifs to form the stable Guanine quartets in vivo and their biological function, for that we pursued a study on the role of G-quadruplex in yeast genome, particularly on the salt tolerance property of yeast. Regulation of salt tolerance genes (GLK1, GPD1, STL1 & YGP1) as they have G4 sequences in their promoter regions; was scrutinized in wild type and evolved strains of Saccharomyces cerevisiae (W-303 &VL-2).G-quadruplex specific binding ligand, NMM, MIX (Non quadruplex binder) at a concentration of 0 µM and 12 µM was used along with 0% and 8% NaCl in YPD media as growth conditions for the yeast strains. Growth curve analysis of these strains of yeast pronounced that the survival percentage of cells has been reduced to ~50% in comparison to control; at a higher concentration of NaCl (8%) and in presence of NMM. Whereas, in the presence of MIX (unmethylated analogue of NMM, which does not bind to G-quadruplex), there was a minimal effect on survivability of yeast indicating that NMM binds to G-quadruplex and may function in stabilizing it so that G- quadruplex can become effective. These results unfolded a notion that decrease in survival percentage in yeast, can be due to the presence of NMM stabilized G-quadruplexes. To ascertain the above finding, the salt tolerance genes containing G4 motifs in their promoter region were analysed by quantitative Real-Time PCR for their expression regulation. Observations clearly showed a down regulation of salt tolerance causing genes indicating G-quadruplex mediated regulation of gene expression. This study thus, exemplifies that G-quadruplex structures can have a role in regulation of the genes in yeast strains indicating their presence in their promoterregion.

Key words:*G-quadruplexReal-Time PCR , DNA, Salt tolerance*

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I. INTRODUCTION:

The most well studied four-stranded structure, and the one that is likely to have the greatest physiological relevance, is the G-quadruplex. It has enticed structural biologists for over a century, but finding its possible biological functions has remained elusive. In 1910 it was reported that concentrated solutions of guanylic acid forms a gel (Bang, *et al*. 1910). During the 1960s, when X-ray diffraction was established as a tool for studying the structure of biomolecules, this gel was found to comprise planar tetramers of guanines connected by Hoogsteen base pairing (Gellert, Lipsett, and Davies 1962). This has been termed a G-quartet or G-tetrad (Balasubramanian and Hurley 2011). Subsequently, a G- quadruplex was found to form also in a control region of the proto-oncogene c-Myc (Simonsson, Pecinka, and Kubista 1998), suggesting a function in transcriptional regulation. Further, indication of role of G- quadruplexes in transcription came through computational studies which found that they are largely absent in exons, but are frequently found upstream of transcription start sites (TSSs) (J. L. Huppert 2008). Recently antibodies were used to visualize G-quadruplexes throughout the mammalian genome (Biffi *et al*. 2013). There is convincing indirect evidences on the importance of G-quadruplexes *in vivo*, acquired through characterization of proteins that resolve or stabilize G-quadruplexes and their functions in living cells.

Eukaryotic cells responds to numerous changes in their vicinity by changing their protein repertoire. Signal transduction pathways involving both transcriptional and post- transcriptional regulatory mechanisms elicits these kind of responses in yeast. The cellular response of yeast to high salinity is studied extensively

hence, serving a model for geneexpression changes in response to salt stress. Literature shows that transcriptional response to high salinity has been nicely approached (Posas *et al*. 2000; Rep *et al*. 2000; Gat-Viks and Shamir 2007) but, the role of G-quadruplexes as a factor of transcription initiation or inhibition has not been catered much to. Yeast cells transiently resists the translation at higher NaCl condition, but it also resumes translation following an adaptation period, which can be directly correlated with the intensity of the saline stress (Uesono and Toh-e 2002). Microarray study conducted by (Arava *et al*. 2003) shows the cellular response of yeast against high salinity. These include genes which are known to contribute to high salt resistance like, *GPD1, HOR2* and *GLK1* that are involved in the biosynthesis and transport of glycerol to counterbalance the high osmotic pressure (Albertyn *et al*. 1994; Pahlman *et al*. 2001) and also, there were some genes whose contribution to high salt resistance is still not clear but their expression was shown to be required for salt tolerance (such as *HMT1, ERG6, YGP1, NCL1*,and YNL168C) (Fernandez-Ricaud *et al*. 2007).

II. Materials and Methods

Culturing of Yeast on Agar plates:

Plating of microorganisms is a traditional microbiological technique. Plating is a qualitative method usually used for separation of mixed cultures and isolating pure cultures. As a matter of fact, it is a dilution technique to spread a loop full of culture on entire plate to separate individualcolonies.

Procedure:

Laminar air flow hood surface was cleaned with 70% alcohol and given UV treatment for 15 minutes to make it contaminationfree.YPD agar was weighed according to the volume required and autoclaved for 20 minutes at 121^0 C and 15 lbs pressure, which was on cooling plated in round petri plates (90mm).After solidification of the plates, streaking was done with a loopfull of culture and the petri plates were sealed with parafilm to prevent contamination.Plates were kept at 30°C for incubation up to 48 hours. After observing for sufficient growth the plates were taken out of the incubator and kept in refrigerator (Once streaked, those plates can be stored up to one month at 4^0 C and then used for further experiments).YPD broth was prepared and taken 15 ml of media in 50 ml falcons. It was later inoculated with a single colony of particular yeast culture for furtherexperiments.The falcon tubes were kept at 30°C in incubator shaker for 18 hours at 200rpm.Each Yeast culture was then checked for Optical density (OD) at 600nm.The optimum OD should be between 0.6-0.8 which shows that the cells are still in log phase of growthcurve.These cultures were used for further experiments like growth curve analysis and RNA isolation.

Growth curve analysis of Yeast in presence of Salt stress and G4ligand:

A growth curve is an empirical style of the evolution of a quantity vs time. Growth curves are widely utilized method in biology for quantities such as population size or biomass. Values as when it comes to measured property can be plotted on a graph as a function of thetime.

Procedure:

Master culture was prepared by taking loop full of culture from agar plate and inoculated in 5ml of YPD broth and incubated for 16 hours to obtain OD of1.0-1.5. Taken different falcon tubes containing YPD broth of 10ml of control and different concentration of NaCl (0%, 6% & 8% NaCl) and added 12µM of NMM and MIX added to the mediumrespectively.100µl of culture was taken from master culture and added in all the culture tubes containing different concentration of NaCl and G-quadruplex bindingligand.After the 0 hour readings were taken by adding culture in 96 well plates to take the OD of cultures at a time and closed the falcon tube with cap and wrapped with parafilm to preventcontamination.Readings were taken in Tecan multi-mode plate reader at 600nm for every 6 hour interval up to 48 hours and OD_{600} was noteddown.Using this OD_{600} value, growth curve of the organism was plotted. (Absorbance versestime).

Procedure:

Relative survival % is calculated by using the growth curve analysisdata. Growth curve was plotted by getting OD values at different intervals of time (0 h, 6 h, 12h…. upto 48h). Survival percentage analysis was calculated according to the growth curve data at different intervals of time with respect to the control growthdata.

Isolation of RNA from Yeast (QiagenKit):

Yeast cells were grown with specific treatments in a 30^0 C shaker incubator until it reaches OD of 0.6-0.8(RNA should be isolated in logphase). Pelleted down the cells by centrifugation at 5000 rpm for 15 min at 4^0 C to prevent cell damage. Carefully decanted the media, without disturbing thepellet. Kept the falcon tubes containing pellet in inverted position to remove anyexcess media, as media may affect the yield ofRNA. Lysis

buffer of pH 8.0 was prepared containing D-Sorbitol, β- Mercaptoethanol (0.1%), 0.1M EDTA and lyticase enzyme (200U) from *Arthrobacter luteus*, to efficiently lyse the yeastcells. Cells were resuspended in 200 µl of Lysis buffer and was homogenized by gentle pipetting and incubated at 30^0 C for 30 min with gentle shaking to degrade the cell wall of yeast, thus generating thespheroplasts. Subsequent to incubation period, to the samples 350 µl of RLT buffer provided by manufacturer added and vortexed vigorously to lyse the spheroplasts. Before using RLT buffer, β- Mercaptoethanol should be added to the RLTbuffer. After lysing the spheroplasts, 250 µl of molecular grade ethanol (100%) was added and mixed well by pipetting the solution to make it into a homogenizedsolution. Immediately, the total sample was transferred onto to an RNeasy spin column provided in kit and centrifuged it for 15sec. At 10,000 rpm and discarded the liquid settled at the bottom of collection tube. Addition of ethanol ensures that RNA binds to the column byprecipitation. In the next step, 700 µl of RW1 buffer was added to the spin column, closed the lid gently and centrifuged the spin column at 10,000rpm for 15 sec, discarded the flow through. RW1 is a wash buffer to clean the spin column to removecontamination. Later 500 µl of RPE buffer was added to the sample and centrifuged at 10,000 rpm for 15 sec and discarded the flowthrough. Repeated the above step again with one modification i.e., centrifugation time was increased upto 2min to dry the column and discarded the flow through along with collectiontube.
Purpose of the long centrifugation is to remove ethanol otherwise it may interfere with the RNA yield in the subsequent steps. The RNeasy spin column was placed in a new eppendorf tube and added 40 µl of Nuclease free water, and incubated for 10min at room temperature, next to the incubation period, centrifuged the spin column at 10,000 rpm for1min. RNA was eluted in eppendorf tube and then stored at -20^0 C for further experiments. The integrity of RNA was checked by running (2 % of gel) agarose gel electrophoresis and Nanodropquantification.

DNase treatment (Ambion TURBO DNaseKIT):

DNase treatment was done according to manufacturer's protocol for removal of DNA contamination from RNA. DNase is an endonuclease that cleaves DNA by breaking phosphodiester bonds. It specifically degrade DNA rather than RNA, thus removing genomic DNA contamination.

Procedure:

Table 1: DNase treatment reaction components and the volume used

Component	Volume (µl)
RNA	Based on the concentration to make 2 µg RNA
10X DNase buffer	6 µl
TURBO rDNase	4 µl
Nuclease Free water	To make total volume to 50 µl

DNaseDigestion:

Two microgram of RNA was used in this reaction and nuclease free water made up volume to 50 µl**.** Subsequently, 6 µl 10X DNase buffer was added to all the samples and 4 µl of TURBO rDNase was next mixed. All the samples were gently mixed followed by short spin to ensure proper mixing of the samples. Afterwards samples were incubated at 37^0 C for 45 min, for every 15 min the samples were vortexed gently followed by short spin and incubation again.

DNaseinactivation:

DNase inactivation reagent is a viscous fluid used to inactivate the DNase after DNase treatment of RNA and also helps in removal of divalent cations like magnesium and calcium, which can catalyse the degradation of RNA at hightemperatures. 5µl of this reagent was added to the samples and incubated at RT for 5 min by occasionalmixing. In the subsequent step, the samples were centrifuged at 8000 rpm for 1 min to pellet down the DNase inactivationreagent. Carefully separated the supernatant containing the RNA and transferred to a new tube without disturbing thepellet. Now the RNA was prior checked for DNA contamination by running the agarose gel and can be used for further experiments and the RNA integrity was checked by Nanodrop spectrophotometer and Agarose gel electrophoresis(2%).

cDNA synthesis fromRNA:

RNA can be converted into cDNA (complementary DNA) by an enzyme called reverse transcriptase. It uses single stranded RNA as a template and synthesizes DNA, a mechanism used by retro viruses as their genetic material is RNA. Conversion of RNA to DNA process is called reverse transcription as it is completely opposite to typical transcription process, i.e., DNA is used as temple and yields RNA.

Preparation of Master mix:

Table 2: cDNA reagents composition

Components	Concentration	Volume per one reaction (µl)
RT Buffer	10X	2.5
Random primers (Hexamers)	10X	2.5
dNTP mix	25 mM	1.25
MULV reverse transcriptase		1.25
Total	-	7.5

Master mix was prepared accordingly as per requirement based on the sample size. A volume for a concentration of 2 µg of RNA was taken and volume was made up to 17.5 µl by adding nuclease free water.7.5 µl of master mix was added to all the samples and mixed gently by vortexing followed by a short spin to settle down reagents to the bottom of the tube. Optimal temperature for enzyme to synthesize cDNA is 37^0C. All the sample were kept in thermocycler to synthesize cDNA.

cDNA cycling conditions:

Table 3: Operating conditions for cDNA synthesis

25^0 C	37^0 C	85^0 C	4^0 C
10 min	**120 min**	**5 min**	∞

After incubation, the cDNA prepared from RNA was checked by doing semi quantitative PCR.

Polymerase chain reaction(PCR):

The polymerase chain reaction (PCR) is a powerful molecular biology technique. It is proficient and quick *in vitro* method for enzymatic amplification of specific DNA sequences of nucleic acids of various sources. Developed in 1984 by Kary Mullis (Barlett & Stirling). A basic PCR setup requires several components and reagents. (Joseph Sambrook and David W. Russel) The components include:A template DNA contains the DNA area (focus/ gene of interest) to be opened up. Primers, which are a pair of short oligonucleotide have their sequence, complementary to both of the targets. *Taq polymerase* or any other polymerase with the temperature ideal at around 70^0C can be used. Deoxynucleoside triphosphates (dNTPs; likewise ordinarily called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a daughter strand. Buffer solution gives a suitable environment for ideal action and stability to the DNA polymerase. Divalent cations, Mg2+ or Mn2+; for the most part Mg2+ is utilized yet Mn2+ can be used for PCR interceded DNA mutagenesis, as higher Mn2+ builds the lapse rate during DNA synthesis.

Amplification

Typically the amplification reactions were done for ~35 cycles, with specific alterations arrived at, empirically to optimize yield. The program was as follows:-

95°C –Initial Denaturation for 5 minutes in the first cycle. 95°C -Denaturation for 30 seconds for all cycles. 58°C-Optimum annealing temperature $T_{a\ OPT}$ for 45 seconds, for all cycles. 72°C -Extension for 40 seconds for all cycles. Cycling for ~30 times. 72°C for 10 minutes to fill the incomplete extension products. 4°C for a minimum of 10 minutes.

Optimizations:

i. Optimization of annealing temperature:

The annealing temperature was calculated using the following parameters according to *Rychliket al,1990.*

$T_{a\ Primer} = 0.3T_{mPrimer} + 0.7\ T_{m\ Product}$

$T_{m\ Product} = 0.41(\%G+C) + 16.6\ (\log_{10}[K^+]) + (675/l) + 81.5\ °C,$

Where, l characterizes the product's length in base pairs (bp), and [K+] speaks to the potassium ion concentration in 1x PCR buffer which is typically given as 50 mM $T_{mPrimer} = 2\times (A+T) + 4 \times (G+C)$, where A, T, G and C are the quantity of the separate nucleotides in the oligonucleotide primer sequence. The Ta OPT was normally taken as the two's lower Ta Primers computed as above. The Ta accordingly landed at, was entirely near the optimum temperature, only a variation of0.7

ii. Optimization ofMgCl2

The Mg++ ion concentration of the amplified reaction was standardized observationally, changing the concentration from 1mM to 4mM in additions of 0.5 mM to get the bestamplification.

iii. Optimization ofyield

Utilizing the above parameters, the amount of template, and the number of cycles were changed accordingly and a perfect mix was achieved to enhance the yield.

PCR components:

Table 5: Master mix composition for PCR reaction set up

S.No.	PCR Reagents	Concentration	Volume for 1 reaction (20 µl)
1	NFW	-	10.9 µl
2	*Taq* Buffer	10X	2.2 µl
3	$MgCl_2$	25 mM	0.6 µl
4	dNTPs	10 mM	1.2 µl
5	Fw primer	10 µM	0.8 µl
6	Rv primer	10 µM	0.8 µl
7	DMSO	100%	1.0 µl
8	*Taq* polymerase	3 U/µl	0.5 µl

Note: DNA or cDNA samples are added separately after addition of master mix to all the PCR tubes.

Agarose gelElectrophoresis:

PCR products were checked on 1% agarose gel using 1X TAE buffer.

Preparing the Agarose gel(1.0%)

100ml of MQ was measured in a graduated cylinder. It was poured into a 250ml flask containing 2g of electrophoresis grade agarose (Sigma Aldrich) and was melted in microwave for 3minutes. It was completely dissolved by slow swirling to make a homogenous solution. 3.0ml 50X TAE buffer was added to the solution to make the final concentration of TAE buffer, 1X. It was swirled and allowed to cool till the temperature reaches about50-60°C. 5µl of EtBr (20mg/ml stock) was added so as to make the final concentration of 0.5µg/ml and it was then mixed by gentleswirling. The comb was placed in the slots on one end of gel casting tray. The agarose gel was placed in a gel tank filled with 1X TAE buffer. The comb was removed by carefully pulling it upwards, upon cooling of thegel.

Loading on thegel

4µl of 6X DNA gel loading dye was added to 20µl of PCR product and mixed by pipetting. The total amount was carefully loaded into the well of the gel.

Running thegel

The positive and negative electrodes were connected appropriately to the power supply. The power supply was switched on and the voltage adjusted to about 90-100 volts.

Visualization ofDNA

After running the PCR product on the gel; the bands were visualized using a UV Gel documentation system.

Real time PCR:

It is also called quantitative real time polymerase chain reaction (qRT PCR). It is a molecular biology method based on the normal principle of polymerase chain reaction (PCR), and is used to amplify and rapidly quantify the given template (DNA or cDNA). Real time qPCR allows both detection and quantification of given template. The magnitude can be either an absolute number of copies or comparative amount based on the reference genes that has been taken as astandard.

Procedure:

Optimization of cDNAdilutions:

2 µg of pure RNA was used to prepare cDNA. Various dilutions of cDNA was used to avoid the undetermined C_T values. In this study cDNA diluted to 30 times has been used.

Master mix preparation:

Table 6: Master mix composition for qRT-PCR

S.No.	Reagent	Concentration	Volume (µl) for 1 reaction
1	SYBR green 1	2X	5
2	Primer pair	0.5 µM	1

3	NFW	-	2
4	cDNA	30 dilutions	2

Note: cDNA was added separately after addition of master mix (8µl) to all the wells of reaction plate.

Reactionsetup:

Prepared enough master mix to run all samples in duplicates and calculated accordingly as per requirement including NTCreactions.For NTC reactions, add 2 µl of water instead of cDNA sample to the reaction well (MicroAmp™ Fast Optical 96-Well ReactionPlate).For experimental reactions, aliquoted 8 µl of master mix followed by 2 µl of cDNA sample to the reactionwell.After addition of master mix and samples the reaction plate was sealed with an optical adhesive cover and vortexed it for proper mixing of reactioncomponents.Centrifuged the reaction plate for a short time and outwardly affirmed that every one of the wells contains sample at the bottom at the right volume with no bubbles.

Note: All the reactions were carried in absence of light because the SYBR is light sensitive.

Cycling parameters for RT-PCR:

Table 7: Optimal temperature conditions for RT PCR

Condition	**Temperature (^{0}C)**	**Time (S)**
Initial denaturation	**95**	**30**
40 cycles		
Step1 (Denaturation)	95	5
Step2 (Annealing)	58	15
Step3 (Extension)	72	10

Prediction of PG4 motifs in the yeastgenome:

By using computational methods, the Quadruplex forming motifs can be predicted on sequences.

Procedure:

The gene of interest sequence was retrieved from yeast genome sequence data base (SGD database) from the website:http://www.yeastgenome.org/cgi-bin/seqTools. A sequence was considered as a potential quadruplex motifs when it satisfies the followingconditions.

a) The string size of a single quadruplex is considered as constant and it can be in the range of 2 to5.
b) String should be made of guanine bases (G) or cytosine (C) on the complementarystrand.
c) The size of loops are between 1 to 7, loops can possess any base (A,G,T &C)
d) A single quadruplex is made of four strings and threeloops.

Single string structure of quadruplex is

$$G_XY_nG_XY_nG_XY_nG_X$$

x– Number G runs it can be between 2 to 5.

Y – It represents loop sequence it can contain any nucleotide base. n – Loop length can be around 1 to 7 bases long.

Any sequence obeys the above formula can have the capability to form G- quadruplex. Upstream sequence of range around 1kb of gene was checked for the presence of G- quadruplex motifs in thesequence.

III. Results and Discussion :

Relative survival (%) of W-303 in presence of NMM & MIX under salt stress condition:

Yeast W-303 strain was grown in YPD broth in the presence of G-quadruplex ligand (NMM) and MIX (non-quadruplex binder) with a concentration of 12 µM. With same concentrations of NMM and MIX, NaCl stress was given at its various concentrations of 0%, 6% & 8%. Growth curve analysis was done upto 48h and readings were taken (OD_{600}) for every 6h interval. Based on the growth curve data, Relative survival (%) of each yeast strain was calculated.

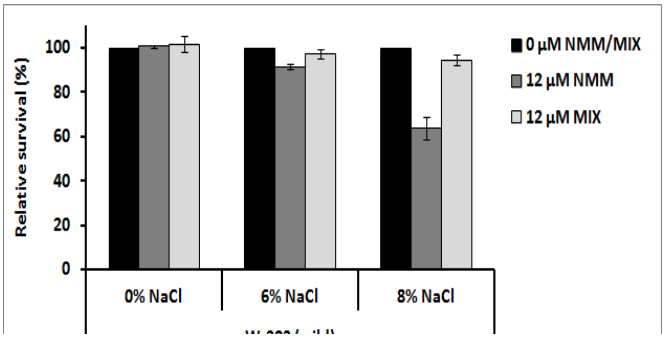

Figure 11: W-303 wild strain relative survival (%) under salt stress and NMM (G4 ligand) and MIX (Negative control).

Above graph represents the relative survival (%) of yeast W-303 strain. The relative survival (%) was calculated based on the respective control, i.e., 0%, 6% & 8% NaCl with 0%, 6% & 8% NaCl treated with NMM and MIX respectively. The relative survival (%) is in decreasing fashion as the NaCl stress increases in presence of NMM in 6% & 8% NaCl. In the control the survivability is 100% but in the NMM treated sample (8% NaCl) the survivability has decreased almost to half (50%), but in MIX treated sample there was no effect on survival percentage. Thus, it indicates that MIX doesn't bind to G-quadruplex, as MIX is unmethylated form of NMM which doesn't bind to G-quadruplex (*Nicoludis et al. 2012).* It suggests that may be the G-quadruplex is compromising the salt tolerance in yeast, because NMM specifically binds to G-quadruplex (*Cahoon et al. 2009),* as in 8% NaCl control (in presence of MIX) the growth of organism was not supressed but only in presence of NMM, it hasdecreased.

Relative survival (%) of W-303 evolved population in presence of NMM & MIX under salt stresscondition:

As discussed above in section 6.1, the procedure followed was same but strains used were evolved form of wild type strain (W-303).

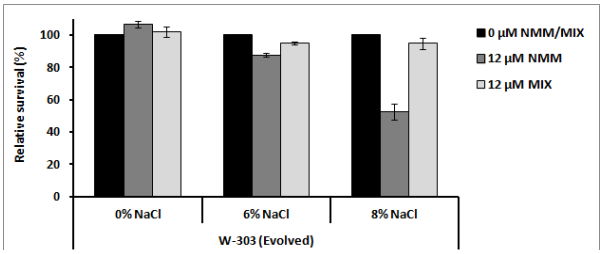

Figure 12: The relative survival (%) of evolved strains of yeast under salt stress conditions.

The evolved strains were continuously grown under increasing amount of salt conditions upto 3000 generations to make them salt tolerant, i.e., the wild type strain was grown in high salt conditions and it transformed into a salt tolerant strain. The relative survival (%) of evolved population was calculated by performing growth curve analysis in presence of NMM and MIX under salt stress. The relative survival (%) was calculated by considering two independently evolved population. The survival (%) was decreased in a fashion as the NaCl concentration increases (only NMM treated condition). But in MIX treated sample there was no evident change in survival percentage. The survival rate was 85% in 6% NaCl (NMM treated) sample and in 8% NaCl treated sample, the survival rate declined to ~50%. This is clearly indicating that G-quadruplex has some role in regulating salt stress inyeast.

Relative survival (%) of VL2 (wild) in presence of NMM & MIX under salt stress condition:

Yeast VL2 strain was grown in YPD broth in presence of G-quadruplex ligand (NMM) and MIX with a concentration of 12 µM, under NaCl (0%, 6% & 8%) stress and growth curve analysis was done upto 48h and readings were taken (OD_{600}) for every 6h interval. Based on the growth curve data, Relative survival (%) was calculated.

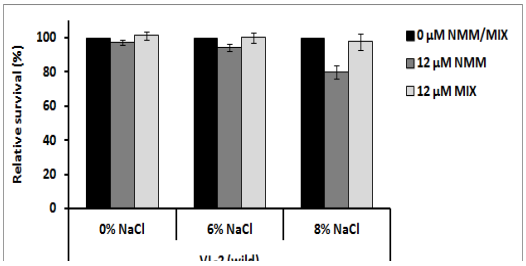

Figure 13: The relative survival % of yeast (VL2) in presence of NMM & MIX under salt stress condition.

The relative survival (%) was calculated based on the growth curve values. The experiment was done at two different concentrations of NaCl (6% & 8%) and in presence of NMM and MIX in the growth media. It was observed that the survival percentage decreased as the NaCl concentration increases in presence of NMM. In this, at 6% NaCl and in presence of NMM, survival rate of yeast has decreased upto approximately 80% and at 8% NaCl treated sample with NMM, the survival rate has decreased remarkably upto 50%, suggesting that G-quadruplex has potential role in affecting the salt tolerance in yeast. Possible way of G-quadruplex affecting the salt tolerance is by modulation of transcription by G- quadruplexes, as the yeast salt tolerance imparting genes contain PG4 motifs in their promoterregions.

Relative survival (%) of VL2 (evolved) in presence of NMM & MIX under salt stresscondition:

In the presence of NMM and high NaCl (8%) concentration, the survival rate of yeast VL2 evolved strains was compromised to 50% but, in absence of NMM and in presence of MIX there was no reduction in growth.Recent findings recommend that G4 motifs are enriched in the genome of yeast, as it has been proved that they form quadruplex structures *in vitro* confirmed by circular dichroism studies and a growing number of proteins have G-quadruplex as substrates, for example yeast Sgs1 p helicase specifically unwinds G4 structures *(Huber, Lee, and Maizels 2002).*

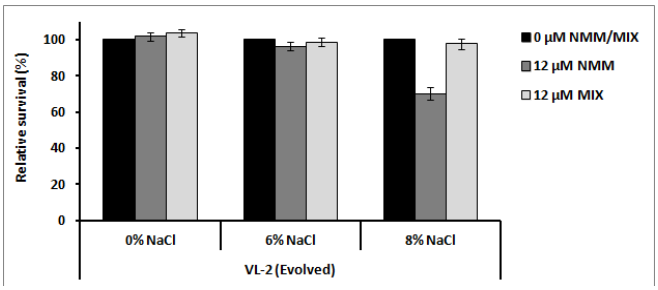

Figure 14: Relative survival (%) of yeast VL2 evolved population.

In any case, envisioning the G4 structures *in vivo* is difficult as they are dynamic in nature yet, they can be targeted by small molecular ligands. A comparable study was done on *Deinococcus radiodurans*, they have demonstrated that G4 motifs are present in the promoter regions of genes imparting radio resistance to the bacterium, for instance Rec family proteins *(Beaume et al. 2013*). Their study implied that G quadruplex can be targeted by small molecular ligands such as NMM (a potent G4 binding ligand), it has also been proved that this ligand enters live cells and binds to G quadruplex structures (*Cahoon et al. 2009).*In presence of NMM the radio resistance of *D. radiodurans* was compromised, suggesting the role of G-quadruplex in gene regulation (*Beaume et al. 2013).*

Based on these findings, in this study we examined the effect of G-quadruplex in yeast genome, for instance salt stress has been used as a phenotypic affect as the salt resistance causing genes have G4 runs in their promoter regions, few of those genes are*GPD1*,*YGP1*,*GLK1* and *STL1*. Also, literature suggests thatsalt stressis best understood in yeast model of eukaryotes. These genes significantly upregulates in response to high salt conditions in yeast (*Yale and Bohnert2001*).Thus, for conducting present study, two different yeast strains (W303 & VL2) and evolved populations (salt tolerant) of the same were taken and grown in high salt condition along with the presence of G4 ligand (NMM) and MIX. Further growth curve analysis was done. Based on this, relative survival % was calculated. In presence of NMM under salt stress the survival % has decreased approximately to 80-90% in 6% NaCl condition and ~50% in (8% NaCl) and in MIX treated cells there was no significant change in survival %. Based on the above findings and observations till now, our study implies that, there could be a possibility that G-quadruplexes can affect the expression of genes, by down regulating them. These observations were further checked for confirming the role of G-quadruplexes at molecular level by analysing the gene expression regulation using Real-Time PCR.

Semi Quantitative PCR:

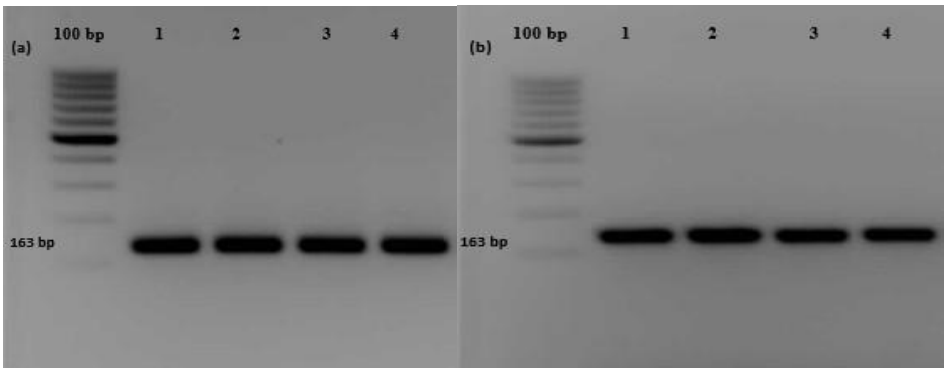

Figure 15: (a) & (b): Semi quant PCR of endogenous control gene (18S rRNA) expression of cDNA samples. In both pictures, sample 1 is 0%NaCl & 0 µM NMM, sample 2 is 0%NaCl & 12 µM NMM, sample 3 represents 8% NaCl & 0µM NMM and sample indicates 8 % NaCl & 12µMNMM.

PCR amplification was done for endogenous control gene (reference gene) or housekeeping gene, with all the cDNA samples. 100 bp ladder was used as the product size is of 163 bp. This experiment was done twice. As it is a semi quantitative PCR and the template used are of same concentration for all the samples, thus amplified product of control gene (RDN18) have got similar intensity in all the samples. For the gene expression analysis the endogenous control gene copy number should be same for all the samples to quantify the geneexpression.

Computational analysis results in prediction of these G-quadruplex structures in promoter regions of genes imparting salt tolerance character to yeast. *GPD1* (YDL022W) contains 5 potential quadruplex motifs in its promoter region in the upstream of 1 kb to the coding sequence (ORF). The quadruplex motifs were present in the positions of 48, 335, 382, 715 789th position of the proximal promoter region. 48, 382 and 789 position havingG runs have high probability of forming the stable G quadruplex. The stability can be predicted by using three conditions; those which are Shorter loops are more common in G runs than longer loops, if a predicted quadruplex sequence have longer loop, then less likely to form a stable quadruplex, another one is G-quadruplex tend to have roughly equal size of loop length in a complete run. Final one is the stability of quadruplex which is directly proportional to the number of G's in a tetrad. *GLK1* (YCL040W) contains 2 PG4 motifs in its promoter region likewise *STL1* (YDR536W) and *YGP1* (YNL160W) contains one PG4 motif in their promoter region. This prediction indicates these genes have PG4 motifs and there is a high probability that they can be formed *in vivo* and their presence can be tested by using specific ligands, as literature suggests that G-quadruplex have the ability to alter the geneexpression.

Gene expression analysis:
Expression of genes by Real time quantitativePCR

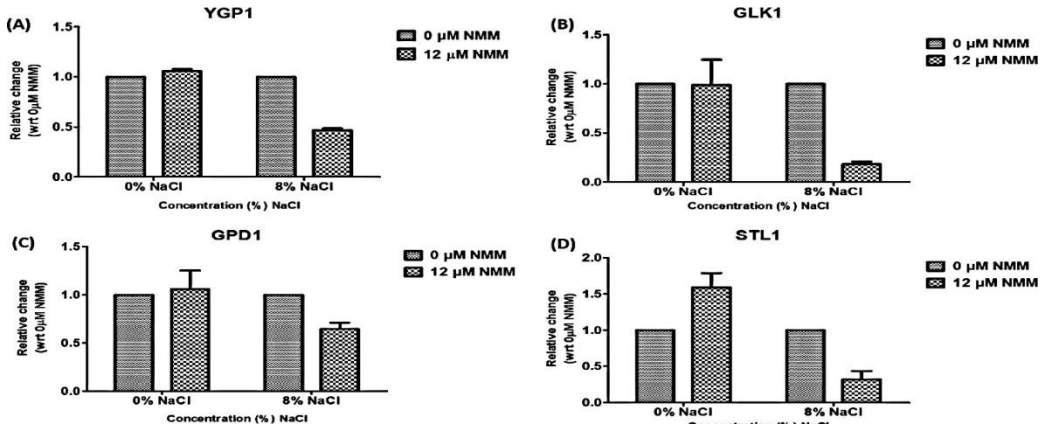

Figure 16: (A): gene expression of *YGP1*, (B). gene expression of *GLK1*, (C). gene expression of *GPD1*, (D). gene expression of*STL1*.

Yeast cultures were grown to mid log phase in YPD media in presence of 12µM NMM under high NaCl condition (6% and 8%) to induce salt stress in the cultures. Absence of NMM ligand was considered

control for the following experiment. Total RNA was isolated from all these treated and control yeast cultures. 2µg of RNA was taken and prepared cDNA followed by DNase treatment, as even small amount of DNA contamination affects the gene expression analysis, after making sure there was no contamination of DNA; cDNA was prepared. The gene expression analysis was done by using real time quantitative PCR technique.Gene expression analysis was done for those genes which have G4 runs in their promoter region as well as gets expressed under salt stress or are known to impart salt resistance in yeast. In this group, one of the genes is *YGP1* (gp37, glycoprotein secreted in response to nutrient limitation), which upregulates in response to nutrient deprivation (*Destruelle, Holzer, and Klionsky 1994*) and a recent study has shown that in response to salt stress this gene upregulates to 25 folds compared to basal level indicating an important role in assisting salt tolerance to yeast (*Yale and Bohnert 2001; Rep et al. 2000*). Here, in the presence of NMM, down regulation of one more key gene in the response to osmotic stress has been clearly observed i.e. *YGP1* (Figure16A). Yeast typical response to osmotic stress is synthesis of osmolytes as they maintain cell volume and fluid balance, an important osmoprotectant is glycerol and yeasts typical response to osmotic stress (*Nevoigt and Stahl et al. 1997*). *GPD1* is key gene for synthesis of glycerol. Yeast strains devoid of the *GPD1* gene shows high sensitivity to salt stress indicating that it is essential for survival of yeast in salinity insult (*Yale and Bohnert et al. 2001). GPD1* also seem to have been down regulated in the presence of NMM and high salt conditions.

Role of ynk1 gene in salttolerance:

Growth curve analysis followed by relative survival (%) was done for wild type, over expressed and mutant strains of yeast (VL2, BY-4741). Relative survival (%) analysed from the above Figure (4.3), indicates, the percentage survivability was high in *ynk1* over expressed strain (VL2) as the NaCl concentration increases from 0% to 3% and finally 6% NaCl.

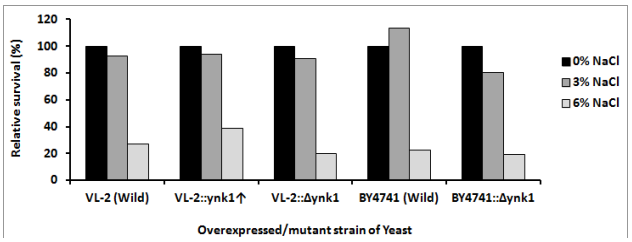

Figure 17: Relative survival % of Yeast over expressed and mutant strains

At high NaCl (6%) the survival rate is high in *ynk1* over expressed strain followed by wild type and mutant strain. It indicates that the over expressed condition of *ynk1* gene promotes the survival of yeast under salt stress. Likewise mutant condition of *ynk1* shows the lesser survival under salt stress in both yeast strains VL-2 and BY-4741 wrt to wild type. Similarly in plants (*A. thaliana*), over expression of ndpk (nucleoside diphosphate kinase) enhances the tolerance of plant to salt stress. As the ndpk is highly conserved (45%) throughout the species (*Lu et al. 1996*), yeast *ynk1* gene also likely to have a significant role in assisting the salt tolerance to yeast.

IV. CONCLUSION:

The study corroborates a prime role of G- quadruplexes in regulating the transcription of genes whose promoter regions accommodates these non-canonical DNA structures. Miscellaneous reports on G-quadruplexes, like on eukaryotic telomerases, in prokaryotes, and yeast have indicated that guanine quadruplexes can influence the regulation of genes by either increasing, repressing their expression. Predominantly, these structures have been seen in the promoter regions of eukaryotic genes and hence vast literature backs the presence and action of G-quadruplexes in various organisms. To understand that whether these informal DNA structures are present without any role or they have assigned any significant character to it, was the main question of our study. In order to answer this mysterious and indispensable question, we chose *S. cerevisiae*, as it has been known to the science as the most dependable, easy and available model organism for diverse category of biological studies. Salt tolerance feature of yeast was the mainstay of this study as it is easily achievable in the lab conditions and also, yeast genome is known to contain various genes which upregulates under salt stress. Here, high salt (NaCl) resistant evolved strains were developed to comprehend the functional identity of G- quadruplexes in the regulation of transcription. Relative survival percentage of these yeast strains under high NaCl and G- quadruplex DNA stabilizing ligand, NMM concentrations along with YPD media as growth conditions for yeast, connoted to the role of these structures in gene regulation, as the growth curve showed approximately 50% decrement in yeast survival. Also, this notion got strengthened by the converging results obtained by quantitative Real- Time PCR exemplifying the significant down regulation of the salt tolerant genes (*GPD1, GLK1, STL1* and *YGP1*) under the above said conditions used for growth curve analysis.

Both the studies evidently manifests that these unique G- quadruplex DNA structures have a tendency toinfluence the regulation of the genes whose promoter sites shelters them. Our study enforces about envisaging the usage of gene regulating property of G-quadruplexes as a therapeutics for various deadlydiseasesacross the world should not be a daydream.

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