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Rice cDNA Encoding PROLM is Capable of Rescuing Salt Sensitive Yeast Phenotypes G19 and Axt3K from Salt Stress

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***Abstract*—**Rice seed expression (cDNA) library in the Lambda Zap 11® phage constructed from the developing grain 10-20 days after flowering was transformed into yeast for functional complementation assays in three salt sensitive yeast mutants *S. cerevisiae* strain CY162, G19 and Axt3K. Transformed cells of G19 and Axt3K with pYES vector with cDNA inserts showed enhance tolerance than those with empty pYes vector. Sequencing of the cDNA inserts revealed that they encode for the putative proteins with the sequence homologous to rice putative protein *PROLM24* (Os06g31070), a prolamin precursor. Expression of this cDNA did not affect yeast growth in absence of salt. Axt3k and G19 strains expressing the *PROLM24* were able to grow upto 400 mM and 600 mM of NaCl respectively. Similarly, Axt3k mutant with *PROLM24* expression showed comparatively higher growth rate in the medium with excess LiCl (50 mM). The observation that expression of *PROLM24* rescued the salt sensitive phenotypes of G19 and Axt3k indicates the existence of a regulatory system that ameliorates the effect of salt stress in the transformed yeast mutants. However, the exact function of the cDNA sequence, which shows partial sequence homology to yeast *UTR1* is not clear. Although UTR1 involved in ferrous uptake and iron homeostasis in yeast cells, there is no evidence to prove its role in Na^+ homeostasis in yeast cells. Absence of transmembrane regions in Os06g31070 protein indicates that salt tolerance is achieved not through the direct functional complementation of the mutant genes but through an alternative mechanism.

***Keywords*—**Rice seed expression, salt stress, prolamin, salinity tolerance, Oryza sativa

I. INTRODUCTION

SALINITY tolerance in rice is a multigenic trait involving a variety of physiological and biochemical pathways. Salinity tolerance in rice varies not only across the cultivars but also across the growth stages. It is relatively salt tolerant during germination, active tillering, and towards maturity, but sensitive during early seedling stage and reproductive stage [1,2]. Partitioning of Na^+ ions alters the concentration of Na^+ ions in the different tissues of the rice plant in growth stage dependant manner. The higher accumulation rates of Na^+ than K^+ in the developing floral parts were observed and may be one of the underlying causes for the sensitivity of the rice to salt stress at panicle induction stage [3]. However, rice grain development was found to be less sensitive to salinity at milking stage [4]. These observations where in the floral parts show higher sensitivity to Na^+ but becomes relatively insensitive during subsequent seed development indicate that developing grain gradually acquire the capability to tolerate salt stress. Hence, studies on the expression of genes and proteins in developing rice seed in response to salt stress could pave the way to identify new salt responsive genes along with novel mechanisms of salt tolerance.

Heterologous expression of plant genes in yeast is a commonly used strategy for functional validation of uncharacterized sequences. *Saccharomyces cerevisiae* L. is a good model system for identifying genes involved in the salt tolerance of plants as both organisms have similar transport systems [5]. Besides, yeast has been extensively studied as a host of heterologous membrane proteins of plants. The mechanisms of ion transport at the plasma membrane and vacuolar membrane are relatively well characterized at the molecular level but the overall homeostatic mechanisms which maintain intracellular ion concentrations are still being elucidated in plants and fungi [6, 7, 8].

Sodium is not essential for most fungi and plants and they are unable to tolerate high NaCl concentrations in soils and water [9, 10]. The major deleterious effects of high salinity in yeast are caused by Na^+ accumulation in the cytoplasm, where many metabolic activities are sensitive to Na^+ inhibition [11]. Influx of Na^+ (and Li^+) in yeast occurs through the K^+ uptake pathway, of which two component transporters were identified as TRK1 and TRK2 [12, 13]. Exposure to high Na^+ content makes this TRK1 and TRK2 systems shifted to a high affinity K^+ uptake system to reduce the Na^+ influx [14]. The efflux of Na^+ is mainly mediated by a putative P-type ATPase encoded by the tandem array of four closely related genes ENA1-4 [15, 16 17]. ENA2, ENA3, and ENA4 present a very weak constitutive expression, while ENA1, although poorly expressed on basal conditions, is induced upon exposure to high concentrations of Li^+ or Na^+, as well as to alkaline pH. Therefore, deletion of ENA1 results in cells hypersensitive to Na^+ or Li^+ [18, 19]. Plasma membrane localized Na^+/H^+ antiport protein, encoded by NHA1, seems to play a role in sodium efflux [20, 21]. This protein though has a little effect on Na^+ efflux, may be important in survival under acidic external pH. In addition to the Na influx and efflux systems, the product of *ScNHX1*, which is homologous to plant *NHX*, has been shown to mediate sequestration of Na^+ within an intracellular compartments, particularly in the vacuole [22].

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Several proteins that are not directly involved with Na^+ and Li^+ transport but modulate the function of monovalent cation transporters in yeast have been identified. HAL4/SAT4 and HAL5, that encode homologous protein kinases are implicated in the regulation of cation uptake. Overexpression of these genes increases the tolerance of yeast cells to sodium and lithium, whereas gene disruptions result in greater cation sensitivity. Hal4 and Hal5 protein kinases are found to be involved in the regulation of TRK1-TRK2 potassium transporters and the stabilization of the transporters in the plasmas membrane [23, 24]. The genes of trehalose pathway , phosphoglucomutase 2 (PGM2) and hexokinase 2 (HXK2) was shown to affect the TRK activity through the modulation of glucose phosphates (glc-1-P and glc-6-P) [25]. A protein phosphatase, calcineurin, has also been reported to be required for the activation of TRK1 and TRK2 in presence as well as absence of sodium stress [14, 26]. Other proteins involved in the regulation of potassium transport include the protein kinase SKY1 [27]; the osmo-induced protein HAL1 [28]; Casen Kinase (CK-2) and G protein of the RAS superfamily, ARL1 [29]. In addition, ENA1 expression is modulated by the HAL3-PPZ1 regulatory subunit-protein phosphatase pair [30, 31]

Considerable progress in understanding plant salt tolerance at a cellular level has been made through heterologous expression studies in yeast. Functional complementation screening of wheat cDNA library led to identify *HKTI* transporter [32]. Screening of cDNA library prepared from 14-day-old seedlings of *Oryza sativa* cv. Akibare identified 1-pyrroline-5-carboxylate (P5C) synthetase (cOsP5CS), an enzyme involved in the biosynthesis of proline [33]. A rice cation transporter gene OsKAT involved with salt stress tolerance was identified through functional complementation of full-length cDNA expression library in salt sensitive G19 phenotype of yeast (*Saccharomyces cerevisiae*) [34]. Salt sensitive yeast mutant for vacuolar antiporter was used in characterising the OsNHX phenotype which is found to play an important role in the compartmentation of Na^+ and K^+ into the vacuoles and confers salt tolerance [35].

The objective of this study is to screen rice cDNA library constructed from developing rice grain (*Oryza sativa* L. variety 'Kinamaze') in heterologous expression system of yeast mutants to identify novel gene candidates involved with salt tolerance. Expression of Rice prolamin precursor protein encoded by Os06g0507200 was found to be capable of conferring salt tolerance in two yeast mutants hypersensitive to salt.

II. Material and Method

A. Amplification of rice seed cDNA library in lambda phage

Rice seed expression (cDNA) library in the Lambda Zap 11® phage constructed from the developing grain 10-20 days after flowering was received from Prof Toshihiro Kumamaru of the Kyushu University, Fukyoka, 812-8581 of Japan.

This rice seed expression library in the Lambda Zap 11® phage was amplified in the *E. coli* strain XL Blue MRF' cells (e14–(McrA–) Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ uvrC umuC::Tn5 (Kanr) lac gyrA96 relA1 thi-1 endA1 λR [F´ proAB lacIqZΔM15] Su–) and the titre was estimated of both the cDNA library and ExAssist® helper subsequently. Efficacy of the cDNA library was determined by blue white colony selection using X-gal and IPTG before carrying out the mass excision. Co-infection of XL-Blue MRF" cells with Lambda phage and ExAssist Helper phage resulted in the in vivo excision of phagemid pBluescript® which is then transformed into the *E. coli* strain SOLR (Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1recA1 gyrA96 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]) for amplification. All these steps were carried out following the protocols given in the Lambda ZAP® II Undigested Vector Kit (Cat no 236201; revision A) from the (Stratagene Europe: www.stratagene.com).

B. Functional complementation assays using Saccharomyces cerevisiae

If Rice cDNA inserts in the pBluescript were subcloned into the Xho1 and EcoR1 restriction sites of pYES2 rice expression vector (Invitrogen, California, USA) under control of the inducible Gal1-promoter [36]. Prior to subcloning, pBluescript vector (3 Kb) backbone was removed by gel purification after digestion with Xho1 and EcoR1 enzymes. cDNA inserts resulted from the gel purification was ligated to pYes using the T4 ligation kit (Fermentas, Maryland, USA) after the dephosphorylation with Antarctic Phosphatase (New England BioLabs, UK). Thus, transformed pYES2 vector was used for yeast functional complementation assays. *S. cerevisiae* strain CY162 (MATa, trk1, trk2::pCK64, his3, ura3), G19 (MATa, his3, ura3, trp1, ade2, and ena1::HIS3::ena4), Axt3K strain (Dena1THIS3Tena4, Dnha1TLEU2, Dnhx1TKanMX4) and the parent strain of G19, W303 (MATa, his3, leu2,ura3, trp1, and ade2), were transformed with the pYES vector with cDNA inserts for complementation assay. Yeast transformations were performed using LiCl method as described by Chen et al. (37). Transformed yeast was plated in Synthetic defined (SD) medium (Galactose 20 g/L, Ammonium Sulphate 5 g/L, Agar bacteriological, 0.67% yeast nitrogen base, 20 g/L adjusted to pH 6) with all amino acids except pyrimidine base uracil with different concentrations of K^+ and Na^+. Histidine was also removed in addition to uracil in the medium that was used for G19 and Axt3K. CY 162 was screened in media containing 0.1 mM KCl upto 50 mM KCl and the control was 100 mM KCl. Axt3K was grown in series dilution of NaCl upto 500 mM, 100 mM LiCl while G19 was grown up to 700 mM NaCl.

C. PCR amplification of the cDNA inserts in the positively complemented clones and sequencing

pYES vector of the yeast clones that showed positive complementation was isolated from yeast using Qiagen plasmid miniprep (Qiagen, West Sussex, UK) protocol after pre-treating the yeast cultures in Zymolase (AMS Biotechnology, Abingdon, U.K.). The isolated plasmids were retransformed into the *E. coli* strain top 10 and cultivated in

the LB- Ampicilline Agar broth overnight in the 27 ^{0}C. Qiagen miniprep™ kit was used to extract plasmid DNA. The cDNA inserts in each vector were amplified with forward (TTCTAATTCGTAGTTTTTCAAGTTCT) and reverse primer(GAAAAAACCCCGGATCGGACTACTAGCAGCTG) flaking the cloning site (94 ^{0}C for 30 seconds, 58 ^{0}C for 30 seconds and 72 ^{0}C for 3 min). The cDNA inserts that showed reliable results after repeated screening in yeast was sequenced for the functional characterization of the cDNA insert at the Biology Technology Facility, University of York. UK.

D.Computer analyses and annotation

To understand and assign functional classes to the sequence information determined in this screen, computational searches were performed against sequence databases at NCBI (http://www.ncbi.nlm.nih.gov/blast) TIGR plant transcript assemblies (http://tigrblast.tigr.org/euk-blast/plantta_blast.cgi) and yeast genome database (http://www.yeastgenome.org) using the BLAST algorithm (Altschul et al., 1990).

III. Results and Discussion

A. Amplification and mass excision of cDNA library from Lambda Zap 11

Rice cDNA library in the Lambda Zap 11 vector system (Stratagene, La Jolla, USA) was constructed from the mRNA of the developing grain of *Oryza sativa* L. variety 'Kinamaze'

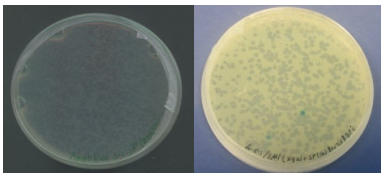

Fig. 1 Estimating the titre and efficacy of the rice seed cDNA library. The *E. coli* strain, XL Blue MRF' (OD_{600} = 0.5) was mixed with Lambda Zap library in 1:1 and 1:10 dilution and incubated at 37 ^{0}C for 12 hours in NZY agar plates. IPTG and X-gal were added to the media for determining the efficacy. Plaque-forming units per milliliter (pfu/ml) was counted after 12 hours of incubation

10-20 days after flowering. Titre and the efficacy of the cDNA library were estimated following the protocols provided in the Stratagene Lambda ZAP 11 manual. Titre of the Lambda phage was 418000 pfu/ml and nearly 99% of efficacy in terms of insertions as was seen with blue white colony selection (Fig 1). These parameters indicated high quality of the cDNA library which used for the screening.

B. Screening rice cDNA library in yeast mutant sensitive to salt stress

Yeast mutants transformed with empty pYES vector and pYES with rice cDNA clones were cultured in the SD – Uracil -Histidine plates with different Na^+ and K^+ concentrations under inducible conditions. Testing for uracil and histidine prototrophy minimize the chance for spontaneous reversion of yeast mutants for tolerance. Of the three mutant strains screened (G19, Axt3K and CY162), growth complementation was seen only in G19 and Axt3K for salt (Na^+) tolerance. Transformed cells of G19 and Axt3K with pYES vector with cDNA inserts showed enhance tolerance than those with empty pYes vector (Fig 2). G19 and Axt3K are found to be sensitive to external Na^+ concentration due to the mutation in genes that encode major Na^+ and Li^+ efflux system. ENA1-4 genes that encode p-type Na^+ pumps are being disrupted in the G19 leading to Na^+ accumulation [38, 39]. Besides the ENA pumps, NHA1 (an antiporter localized in the plasma membrane) and NHX1 (the antiporter that localized in tonoplast and sequestrate Na^+ in the vacuole) are disrupted in Axt3K clone [40, 41, 42]. Hence, Axt3K clone tends to accumulate more Na^+ than the G19 rendering it more salt sensitive than the G19. There were several positive clones of both G19 and Axt3k with enhanced tolerance to salt stress (Fig 3). Positive clones that showed growth complementation at high Na^+ concentrations were retransformed into the same yeast mutant background and rescreened in the higher Na^+ concentrations to confirm the growth complementation.

This screening led to identify a consistent salt tolerant clone in Axt3k backgrounds. Sequencing of the cDNA inserts revealed that they encode for the putative proteins with sequence homologous to rice putative protein PROLM24, a prolamin precursor (BLASTN at MSU Osa1 Release 6.1).

C.Rice cDNA encoding for prolamin precursor PROLM24 confers salt tolerance in salt sensitive yeast mutants

Expression of cDNA clone of PROLM24 conferred tolerance to salt stress induced by NaCl in both Axt3K and G19 yeast mutants compared with the control strains transformed with the empty pYES2 vector.

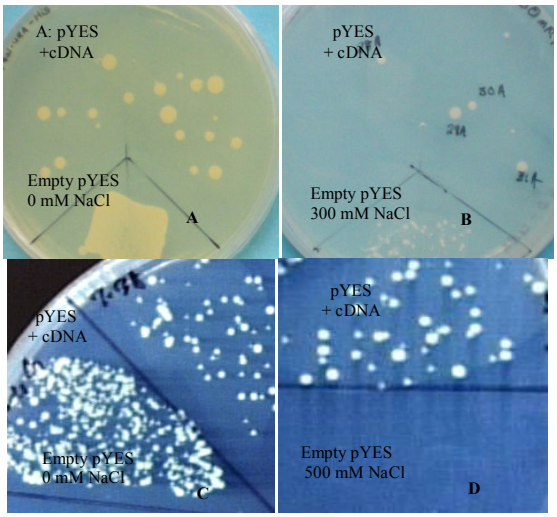

Fig. 2 Screening yeast clone Axt3k (A and B) and G19 (C and D) transformed with rice cDNA (pYES+cDNA) and empty pYES vector for the salt stress tolerance. There were few positive clones of Axt3K appeared at 300 mM Na^+ (upper B) and several positive clones of G19 at 500 mM Na^+ (right D). Growth of the colonies carrying empty vector of both Axt3K and G19 had been severely affected at these Na^+ concentrations

However, expression of this cDNA did not affect yeast growth in absence of salt. Axt3k and G19 strains expressing the PROLM24 were able to grow upto 400 mM and 600 mM of NaCl respectively (Fig 3). These concentrations are well beyond the maximal salt concentration tolerated by wild-type yeast cells in SD medium (data not shown). Similarly, Axt3k mutant with PROLM24 expression showed higher growth rate in the medium with excess LiCl (50 mM) (Fig 4). Transport of Li^+ across the plasma membrane is mediated by the same transport system for Na^+ in yeast [18, 43]. However Li is more toxic than the Na^+, hence cells survived only upto 50 mM LiCl.

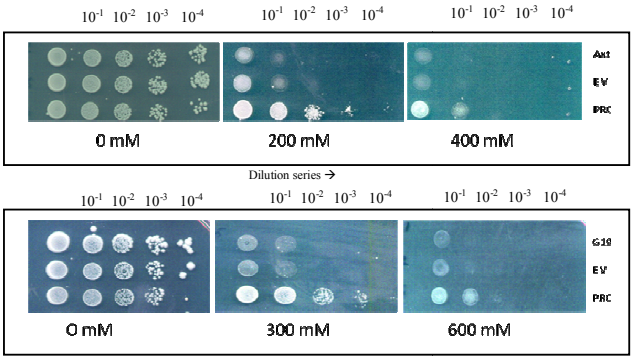

Fig. 3 Complementation of yeast mutants by expression of PROLM24: Growth of the salt-sensitive yeast strain (Axt3K), Axt3K transformed with either the empty plasmid pYES (EV) or PROLM24 expression plasmid (PROLM). B: Growth of the salt-sensitive yeast strain (G19), G19 transformed with either the empty plasmid pYES (EV) or PROLM expression plasmid (PROLM). Drop assay of tenfold serial dilution of yeast cell suspension cultures in the SC-Uracil-Histidine with different NaCl concentrations. Growth was followed for 5 days

The observation that expression of PROLM24 rescued the salt sensitive phenotypes of G19 and Axt3k indicates the existence of a regulatory system that ameliorates the effect of salt stress in the transformed yeast mutants. As mentioned above, salt stress tolerance is dependent on the monovalent ion homeostasis which is maintained by Na^+ uptake and efflux systems in yeast which are represented by TRK1-2 and ENA1-4 genes [12, 13, 15, 18] mainly. In addition, the function of proteins CK-2, HAL3 and PPZ1 [31, 44] and the calcineurin [14] showed to have been involved in salt tolerance in yeast. Study with the expression of CK-2 revealed that the salt sensitivity of the ckb1 mutant (CK-2) is independent of Na^+ fluxes and hypothesized to be involved with protein dephosphorylation [44].

In absence of any evidence for probable function of PROLM24 in the yeast, a sequence similarity search of the cDNA insert (PROLM24) was carried out with BLAST tool against the yeast nucleotide and protein database (www.yeastgenome.org) to identify if there were any functionally similar proteins in yeast.

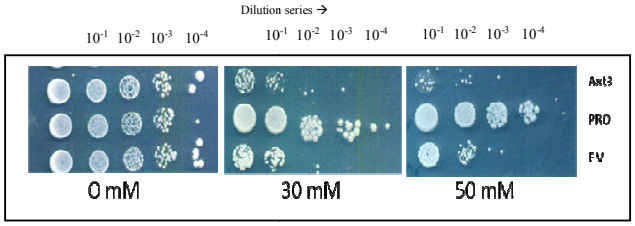

Fig. 4 Complementation of yeast mutants by expression of PROLM24. Growth of the salt-sensitive yeast strain (Axt3k) transformed with either the empty plasmid pYES (EV) or PROLM24 expression plasmid (PROLM) in LiCl. Drop assay of tenfold serial dilution of yeast cell suspension cultures in the SC-Uracil-Histidine with different LiCl concentrations. Growth was followed for 5 days

The result showed that PROLAM24 is partially homologous to yeast protein YJR049C, which encode for ATP-NAD kinases (EC 2.7.1.23) called UTR1 [45] (Fig. 5). Expression of cDNA sequence of heterologous protein PROLAM24 which has partial homology to nucleotide sequence of YJR049C may modify the expression of YJR049C and its protein UTR1 [46].

However, there is no evidence to establish any direct relationship between UTR1 and monovalent cation transport as it has no predictable transmembrane domains [47]. Nevertheless, UTR1 hardly have any effect on modulating the expression or activity of the ENA 1-4 or TRK transporters in G19 and Axt3K mutant backgrounds. Given that there is no significant difference in the intra-cellular Na^+ / K^+ ion ratio between wild type and transformed mutant cells (data not shown), probably the rice PROLM24 (Os06g31070) in yeast may have a function similar to that of Casein Kinase 2 which renders proteins insensitive to Na^+ and Li^+ toxicity [44].

D.Differential regulation of PROLM24 gene in rice

Prolamins are principal storage proteins of rice and so far a total of 34 gene copies located on chromosomes 3, 5, 6, 7, 11 and 12 have been identified in the rice cultivar Nipponbare [48]. Prolamin proteins are not located inside starch granules but primarily located on the exterior of starch granule. Prolamin protein makes a vitreous hydrophobic matrix by cross-linking with starch.

Blasting with NCBI transcript reference sequence database revealed that sequence of cDNA insert is homologous to Os06g31070 (Os06g0507200) of *Oryza sativa* L. [Japonica Group].

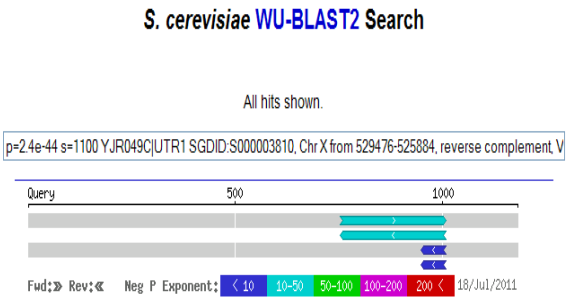

Fig. 5 Sequence comparison of rice PROLM24 cDNA in yeast genome. Prolamin cDNA sequence was compared using BLASTN tool in Saccharomyces Genome Database (www.yeastgenome.org) which showed high partial homology to YJR049C

The protein encoded by this gene is annotated as prolamin precursor which is alpha amaylase inhibitor (AAI) found in the seed storage protein. Generally, AAIs play an important role in the natural defenses of plants against insects and pathogens such as fungi, bacteria and viruses by impeding the digestion of plant starch and proteins by inhibiting digestive alpha-amylases and proteinases [49]. This gene has significantly high expression in rice panicle [50].

However, genome wide transcriptomic studies showed differential expression in the different tissues of the salt tolerant and sensitive rice cultivars in response to salinity stress. Pandit et al [51] recorded that Os06g31070 was differentially regulated in response to salt stress in the recombinant inbred lines (RIL) s derived from the cross between salt sensitive rice cultivar MI 18 and tolerant CSR 27. Salt tolerant RIL showed downregulation of Os06g31070 where as sensitive counterparts showed upregulation. Genome wide expression study at seedling stage revealed that this gene was down regulated in the shoot tissues of both salt sensitive IR 29 and tolerant FL478 in response to salt stress; but more downregulation was seen in the IR 29 [52]. Interestingly, the expression pattern of this gene in the root, at the transcriptomic level, showed upregulation in the salt tolerant FL478 while it was down regulated in the sensitive IR 29 [53]. On the contrary, in the same study of Cotsaftis et al [53] on salt tolerant land race pokkali, showed downregulation of Os06g31070 in response to salt stress. Genome wide root transcriptomic analysis of Senadheera et al [54] on FL 478 and IR 29 showed no significant differential regulation in response to salt stress. A comparative proteomic study in response to salt stress found that prolamin (PPROL 14) precursor was upregulated in salt tolerant Pokkali until 12th hour of treatment while this protein was down-regulated in IR29 [55].

IV. Conclusion

Number Expression of cDNA sequence of the rice prolamine precursor gene PROLM24; Os06g31070 (Os06g0507200) rescued the salt sensitive yeast mutants G19 and Axt3K from the salt stress. Transformed G19 and Axt3K yeast mutants were capable of growing upto 600 mM and 400 mM of NaCl concentration respectively. However, the exact function of the cDNA sequence, which shows partial sequence homology to yeast NAD kinase, UTR1 in the heterologous system, is not clear. Absence of transmembrane regions in Os06g31070 protein indicates that salt tolerance is achieved not through the direct functional complementation of the mutant genes but through an alternative mechanism. Absence of consistent data for salt responsive expression of Os06g31070 in rice makes salt responsive functional characterization of the gene further difficult. The available expression data demonstrate weak correlation with the differential sensitivity of rice cultivars to salt stress. One of the reasons for this inconsistency would be the differences in tissues, stages of growth and growth conditions of the transcriptomic studies used to derive these data. Fairly consistent salt stress responsive differential expression in shoot and root of tolerant rice cultivar FL 478 and sensitive IR 29 indicates that there is a salt associated differential expression of this particular protein at whole plant level [52, 53]. In spite of the shroud of discrepancy on the exact mechanism of PROLM24 (Os06g31070) in rice salt tolerance, there is strong evidence for the relationship between the Os06g31070 and salt tolerance of yeast mutants G19 and Axt3K. Specific study of the expression of this gene in developing panicle in response to salt stress is imperative for the functional characterization of Os06g31070 in rice. Further studies are required to elucidate exact mechanism of action which may points to a novel pathway of salt tolerance in rice.

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