

Introgression of a novel salt-tolerant L-*myo*-inositol 1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka (*PcINO1*) confers salt tolerance to evolutionary diverse organisms

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Abstract We have previously demonstrated that introgression of *PcINO1* gene from *Porteresia coarctata* (Roxb.) Tateoka, coding for a novel salt-tolerant L-*myo*-inositol 1-phosphate synthase (MIPS) protein, confers salt tolerance to transgenic tobacco plants (Majee, M., Maitra, S., Dastidar, K.G., Pattnaik, S., Chatterjee, A., Hait, N.C., Das, K.P. and Majumder, A.L. (2004) A novel salt-tolerant L-*myo*-inositol-1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka, a halophytic wild rice: molecular cloning, bacterial overexpression, characterization, and functional introgression into tobacco-conferring salt-tolerance phenotype. *J. Biol. Chem.* 279, 28539–28552). In this communication we have shown that functional introgression of the *PcINO1* gene confers salt-tolerance to evolutionary diverse organisms from prokaryotes to eukaryotes including crop plants albeit to a variable extent. A direct correlation between unabated increased synthesis of inositol under salinity stress by the *PcINO1* gene product and salt tolerance has been demonstrated for all the systems pointing towards the universality of the application across evolutionary divergent taxa. © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Salinity stress; MIPS; Inositol; Transgenics; *PcINO1* gene

1. Introduction

Salinity presents a multifold challenge to all organisms in terms of perturbed osmotic balance, ionic disequilibria and generation of toxic metabolites and is a major abiotic stress affecting the biological systems particularly limiting plant productivity worldwide [1]. All organisms exhibit characteristic responses towards such abiotic stress effects and possess means to combat these. Depending upon the genetic make-up of the organism and the environment it normally dwells in, these responses vary greatly although some reactions seem to be universal for steady survival in salt-environment. Plant salt tolerance is known to be a multigenic

trait [2] and classical breeding for developing salt tolerance in crops have been attempted with limited success [3]. Genetic engineering of several metabolic pathways producing osmoprotectants/osmolytes or sugars [4–10], regulation of ion homeostasis or other salt tolerance determinants like vacuolar transporters like AtNHX1 [11], transcriptional activators and regulatory genes for raising salt-tolerant plants have earlier been reported by several researchers [1]. However, a well documented mechanism to balance osmotic pressure within the cell and to protect the biomolecules from downward effects has been the increased production of osmoprotectants/osmolytes. Increased production of a number of osmolytes such as proline [4] and glycine-betaine [5], sugar alcohols such as mannitol [6,7], sorbitol [8] and pinitol [9], or a sugar like trehalose [10] have been reported to confer salt tolerance to several groups of plants.

Inositol, an ubiquitous six-carbon cyclohexane hexitol, and its methylated derivative pinitol are implicated in stress responses having a role as osmolytes in several biological systems [8]. In addition to such function, inositol and its derivatives are also implicated in a variety of other cellular processes including growth regulation, membrane biogenesis, signal transduction, ion channel physiology and membrane dynamics [12]. In all inositol producing organisms studied to date this important cyclitol is produced via conversion of glucose-6 phosphate to inositol-1 phosphate by the L-*myo*-inositol 1-phosphate synthase (MIPS; EC 5.5.1.4) involving NAD⁺ followed by dephosphorylation catalyzed by inositol monophosphatase (IMPase; EC 3.1.3.25) [13]. The MIPS enzyme or its structural gene *INO1* has been reported from diverse sources, including higher plants and animals, parasites, fungi, green algae, bacteria and archaea, and has been considered to be an ancient protein/gene [13].

Because inositol is able to protect cells from effects of osmotic imbalance caused by high salinity, search for a positive correlation between increase in the cellular inositol pool and growth and survival of organisms during salt stress is worth investigation. A prerequisite of this is unabated production of inositol under salt stress by a salt-tolerant inositol biosynthetic machinery operative in the surviving system. We have previously reported an *INO1* gene from *Porteresia coarctata* (Roxb.) Tateoka (*PcINO1*), a halophytic wild rice, that codes for a unique salt-tolerant MIPS enzyme. The corresponding homologue, termed *OsINO1* from the cultivated rice, *Oryza sativa*, however, codes for a salt-sensitive MIPS protein [14].

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An insight into the molecular mechanism of salt tolerance of *PcINO1* and the importance of a unique 37 amino acid stretch in conferring the aforesaid trait has recently been documented [15]. Further, *PcINO1* transformed tobacco plants were able to grow in 200–300 mM NaCl with unabated photosynthetic competence and a 2–7-fold increase in cellular level of inositol compared to the corresponding control plants [14]. Because of the ubiquitous presence of inositol throughout the biological kingdom, it was thought pertinent to address the question as to whether increased synthesis of inositol under the influence of *PcINO1* gene can effectively confer salt tolerance to organisms distantly placed in the lineage of evolution.

2. Materials and methods

2.1. Transformation of *E. coli* and their growth pattern in presence of salt

E. coli BL21 (DE3), transformed with the vector pT7-7 (obtained from Dr. S. Dasgupta, Department of Microbiology, Bose Institute), constructs containing *OsINO1* and *PcINO1* genes and no insert were grown in 5 ml culture at 37 °C, induced with 1 mM IPTG and further grown for 2 h ($A_{600\text{nm}} = 0.45$) at 22 ± 2 °C. Finally, equal amount of inoculum, determined by turbidometric method [16] was spread onto solid Luria Broth containing 0–400 mM NaCl containing 1 mM IPTG, under ampicillin selection (100 µg/ml) and incubated at 22 ± 2 °C for 24 h. The growth of the cells in each set was measured quantitatively by count of the colony forming units (cfu).

2.2. Protein expression, purification and checking for in vitro salt tolerance

For checking protein expression, all transformed *E. coli* BL21 (DE3) cells were grown at 37 °C up to $A_{600} = 0.3$ and induced as described above. 500 mM NaCl was added to each culture and the cells further grown overnight at 22 ± 2 °C. After 14 h, ~30 µg protein from each set was analyzed in 10% SDS-PAGE [17] and immunodetected using polyclonal anti-OsMIPS antibody. For determining MIPS activity in presence of salt, overexpressed OsMIPS and PcMIPS proteins were isolated and purified from transformed *E. coli* BL21 (DE3) cultures following the three-step purification protocol described elsewhere [14]. In vitro MIPS activity and salt tolerance property from system was measured [14] with ~25 µg of purified proteins in presence of 0–500 mM NaCl.

2.3. Transformation of *Schizosaccharomyces pombe* and their growth in presence of salt

OsINO1 and *PcINO1* genes were cloned in the yeast expression vector pREP1 downstream to thiamine repressible promoter (nmt) using *NdeI* and *SalI* sites. *Schizosaccharomyces pombe* PR109 (h-ura4-D18 leu1-32) cells were transformed [18] with pREP1 constructs and the transformants were picked up by leu prototrophy. For checking growth profile in presence of salt, transformants were first grown in liquid EMM [19] media supplemented with 2 µM inositol at 30 °C for ~24 h ($OD_{600} = 0.6$ – 0.7). Thereafter, equal volume of inoculum, as determined by the hemocytometer counting of cells, was allowed to grow at 30 °C onto both solid and liquid EMM media supplemented with 2 µM inositol at different NaCl concentrations (0–500 mM). Growth was monitored for 72 and 50 h, respectively by counting the number of viable cells forming colonies (cfu).

2.4. Expression and purification of MIPS proteins from *S. pombe* and their enzymatic activity in presence of NaCl

To check for MIPS expression total protein was extracted [20] from the transformed cells, run on a 10% SDS-PAGE [17], and expression was confirmed by Western blotting with polyclonal anti-OsMIPS antibody. MIPS was purified from the total protein by 35%–65% ammonium sulfate fractionation followed by gel filtration chromatography through Superose-12 [14]. Purified MIPS proteins (~20 µg) were assayed for enzyme activity [14] in presence of increasing salt concentration (0–500 mM).

2.5. Construction of vectors for plant transformation and raising of transgenic *Oryza* and *Brassica* plants

OsINO1 and *PcINO1* coding sequences were cloned at *XbaI* and *KpnI* site of pCAMBIA1301 under CaMV35s promoter and Nos terminator and mobilized in *Agrobacterium* sp. strain LBA4404 [14,21]. Scutellar calli derived from *Oryza sativa* var. Pusa Basmati 1 seeds and *Brassica juncea* var. B85 hypocotyl explants were transformed following standardized protocol [22,23]. Hygromycin selected T₀ plants were grown at 70% humidity and 16 h:8 h photoperiod in hormone free MS medium [24]. Seven independently transformed hygromycin selected and PCR positive *PcINO1* transformed (six copies each of 7 T₀ lines namely P3, P8, P9, P10, P12, P20 and P22) and *OsINO1* transformed (six copies each of 8 T₀ lines namely R5, R6, R7, R9, R12, R16, R19 and R20) and three sets of untransformed in vitro regenerated *Oryza* plants (var. Pusa Basmati 1) were tested by growing them in presence of 0, 50, 100, 200, 250, 300 mM NaCl. Similarly, six independently transformed, hygromycin selected and PCR positive *PcINO1* transformed (10 copies each of 6 T₀ lines namely, Pb2, Pb4, Pb9, Pb11, Pb19 and Pb21) and *OsINO1* transformed (10 copies each of 6 T₀ lines namely, Rb2, Rb6, Rb12, Rb15, Rb19 and Rb25) and three sets of untransformed *Brassica* plants were tested. The single transformants for each crop were selected for experimentation based on the Mendelian segregation pattern for the transgene and the *hptII* gene.

2.6. Molecular analysis of transgenics

DNA was isolated by modified CTAB method [25]. Transformants were confirmed by PCR for *hptII* gene using 5' ATG AAA AAG CCT GAA CTC ACC GCG 3' forward and 5'CTA TTT CTT TGC CCT CGG ACG AGT 3' reverse primers. Introgression of *PcINO1* was confirmed using *PcINO1* specific primers, designed based on the difference between *OsINO1* and *PcINO1* at the nucleotide level e.g. forward (from bp 526 to bp 547): 5'CTC TCC CTG GCA TCT ATG ATC C 3' and reverse primer (bp 956 to bp 933): 5' CCG GTT TTT TTT GGT TTG CCC 3'. Histochemical *GUS* assay [26] was performed using calli or leaf segments from the putative T₀ transformants. Southern blot for *PcINO1* gene was performed according to standard protocol [27].

2.7. Growth pattern and MIPS assay of transgenic plants in NaCl

Eight independent *PcINO1*- and *OsINO1* transformed Pusa Basmati 1 plants and six independent transformed *Brassica* plants along with untransformed control plants were grown in presence of increasing NaCl in hormone free MS media for 21 or 10 days. Cytosolic MIPS from leaves of transformed and control *Oryza* and *Brassica* plants was isolated and purified [14]. MIPS activity was assayed in increasing NaCl concentration using ~15 µg of purified protein.

2.8. Measurement of photosynthetic efficiency

Photosynthetic efficiency of plants during growth in increasing NaCl concentration was calculated by measuring chlorophyll *a* fluorescence using a Plant Efficiency Analyzer (Hansatech Instruments Ltd., King's Lynn, UK) using a single flash of light intensity of 3000 µmol/m²/s after 10 min dark adaptation using Hansatech Biolyzer software. Each chlorophyll *a* fluorescence transient O-J-I-P was analyzed according to the JIP-test [28].

2.9. Determination of inositol content

Leaf inositol content was measured through gas liquid chromatography (GLC) as described by Majee et al. [14] following the method of Bielecki and Redgwell (1977). GLC was done in a Chemito 1000 GC equipped with flame ionization detector. Trimethylsilyl (TMS) derivatives of the samples were prepared with Tri-Sil- Z (Pierce) and were run through 3% SP-2100 stationary phase (Supelco) supported on Chromosorb-W (Sigma). Quantification was made against similar runs with authentic *myo*-inositol as standard.

3. Results and discussion

To evaluate whether *PcINO1* can confer salt tolerance to widely diverse organisms, the gene was introgressed and

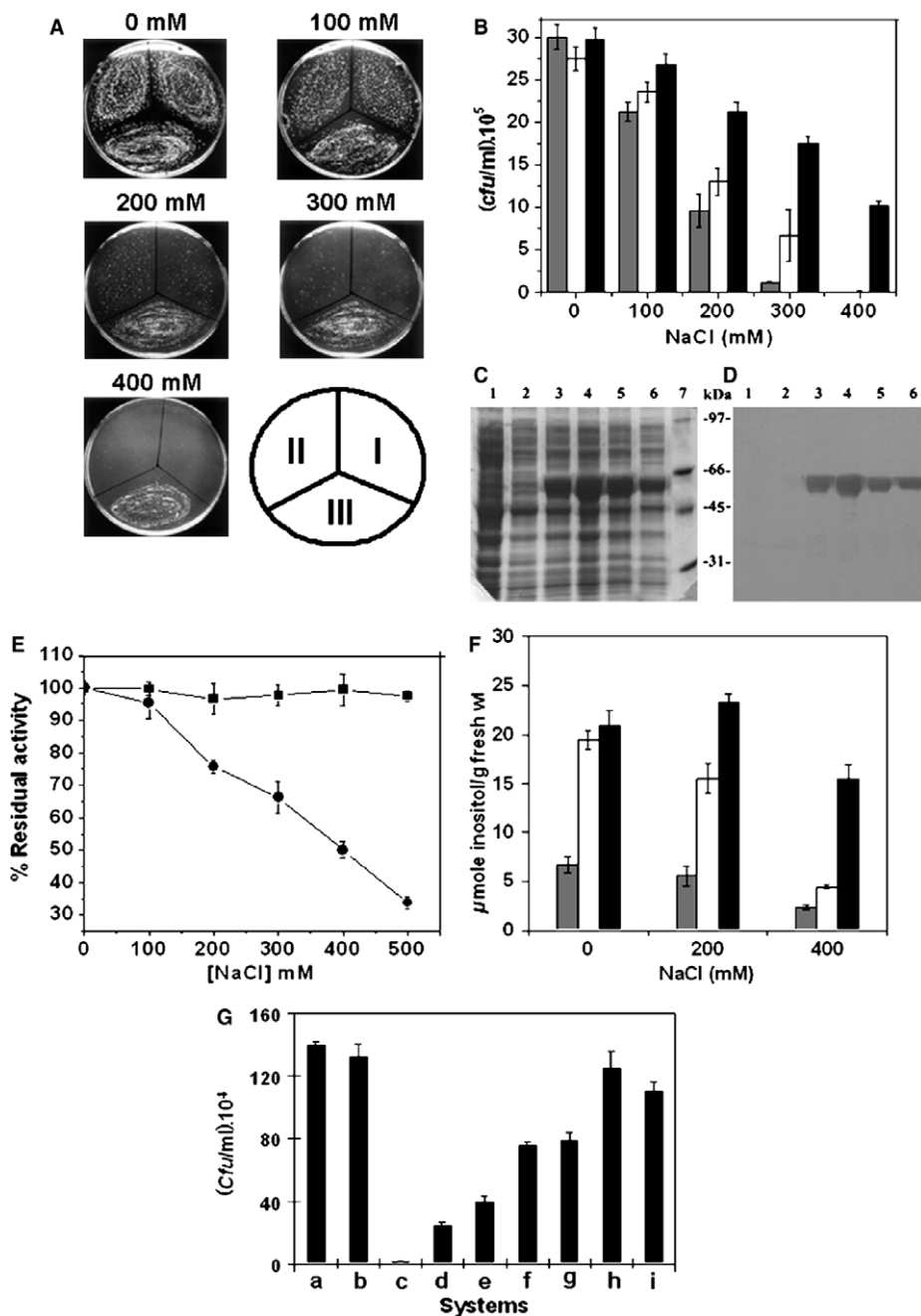


Fig. 1. (A and B) Protection of salt induced growth inhibition of *E. coli* by *PcINO1* expression. (A) Growth of *E. coli* BL21(DE3) transformed with pT7-7 (I), *OsINO1* (II) and *PcINO1* (III) on solid LB agar, supplemented with 100 μ g/ml ampicillin under IPTG induction and in absence or presence of various concentrations of NaCl at 22 \pm 2 $^{\circ}$ C. (B) Quantitative estimation of survivability of *E. coli* BL21 (DE3) transformed with pT7-7, *OsINO1* and *PcINO1* in presence of increasing NaCl concentration. Cell survivability is expressed in terms of colony forming unit/ml under induced condition, in presence of 0, 200 and 400 mM NaCl. Grey bar-empty vector (pT7-7), white bar-*OsINO1* and black bar-*PcINO1* transformed cells. Error bars indicate standard deviation from triplicate experiments. (C and D) MIPS protein expression and immunodetection in transformed cells 10% SDS-PAGE (C) and corresponding Western blot analysis using anti *Os*-MIPS antibody (D) of the protein extract from IPTG induced *E. coli* BL21 (DE3) cells transformed with empty pT7-7 vector, *OsINO1* and *PcINO1* and grown overnight at 22 \pm 2 $^{\circ}$ C in absence of NaCl (lanes 1, 3 and 5) and in presence of 400 mM NaCl (lanes 2, 4 and 6). Lane 7 in (C) represents MW markers. Each lane contains \sim 30 μ g total protein. (E) MIPS activity of *PcINO1* and *OsINO1* transformed salt treated cultures. Enzyme activity assay of purified MIPS isolated from IPTG induced *E. coli* BL21(DE3) cultures in presence of increasing concentration of NaCl in vitro. *E. coli* cells transformed with *OsINO1* and *PcINO1* were grown in 400 mM NaCl overnight at 22 \pm 2 $^{\circ}$ C. MIPS activity from *PcINO1* (\blacksquare -) and *OsINO1* (\bullet -) transformed cells are designated. (F) *Myo*-inositol content of salt treated cultures. Free *myo*-inositol content of *E. coli* BL21(DE3) cultures expressing *PcINO1* and *OsINO1* both under salt stressed and unstressed condition. Amount of *myo*-inositol has been expressed in terms of μ mole inositol/gm of fresh weight of cells. Error bars indicate standard deviation from three independent estimations. Grey bar-empty vector (pT7-7), white bar-*OsINO1* and black bar-*PcINO1* transformed cells. (G) Protection of salt induced growth inhibition of untransformed *E. coli* BL21(DE3) in presence of 400 mM NaCl by different concentrations of *myo*-inositol. Cell survivability is expressed in terms of colony forming units/ml. Error bars indicate standard deviation from triplicate experiments. Different systems containing salt and inositol have been numbered below each lane. Lane a, 0 mM NaCl + 0 mM inositol; lane b, 100 mM inositol; lane c, 400 mM NaCl; lane d, 400 mM NaCl + 10 mM inositol; lane e, 400 mM NaCl + 20 mM inositol; lane f, 400 mM NaCl + 40 mM inositol; lane g, 400 mM NaCl + 60 mM inositol; lane h, 400 mM NaCl + 80 mM inositol and lane i, 400 mM NaCl + 100 mM inositol. All experiments have been performed with three independently transformed *E. coli* cells lines.

tested for functional expression in four distantly related organisms from unicellular prokaryote *E. coli*, unicellular non-photosynthetic eukaryote *Schizosaccharomyces pombe*, a monocotyledonous crop, rice (*Oryza sativa*) and a dicotyledonous oil-yielding crop, mustard (*Brassica juncea*). *E. coli*, besides serving as the prokaryotic model, seemed to us very relevant for this study because it inherently lacks an MIPS protein although contains *subB*, a homologue of IMPase [29]. *Schizosaccharomyces pombe* is considered a natural inositol auxotroph lacking the MIPS coding gene despite harboring an IMPase activity [30] and requires external inositol for its normal growth. Thus the organism presents an ideal model for studying the growth of the transformed cells in increasing salt concentration under the influence of a salt-tolerant MIPS. The *INO1* genes from two experimental crop plants such as *Oryza sativa*, (GenBank Accession number AB012107) and *Brassica* sp. (GenBank Accession number U66307) are known to code for salt-sensitive MIPS proteins.

Although there are earlier reports of glycine-betaine, proline, triethylamine N-oxide (TMAO), trehalose, potassium glutamate and glycerol [31] acting as compatible solutes in *E. coli*, the role of inositol as osmoprotectant in this bacterium, inherently lacking an MIPS protein, was yet to be ascertained. In order to assess this, *E. coli* BL21 (DE3) cells were transformed with the empty vector pT7-7, vector constructs containing either *OsINO1* or the *PcINO1* gene. Three independent groups of transformed cell lines were used for experimentation. Growth of such cells under IPTG induction was measured on solid LB media with increasing NaCl in absence of externally supplied inositol. The transformed cells grew almost to the same extent at 100 mM NaCl although with increasing NaCl in the growth media, a steady inhibition of growth was observed in case of *OsINO1* and empty pT7-7 vector transformed cells with very little growth inhibition for *PcINO1* transformed cells after 24 h of incubation (Fig. 1A). Such growth pattern and survival of cells under conditions of increasing salinity were quantitated by tracking cell growth using plating and colony counts (Fig. 1B). As apparent, between 200 and 400 mM NaCl the *PcINO1* transformed cells show better survival in comparison to the other two cell types. Although extent of growth or survivability was reduced even in case of *PcINO1* transformed cells at 400 mM NaCl (about 40%), in case of *OsINO1* and empty vector transformants, virtually no growth was observed at this salt concentration (Fig. 1A and B).

Expression of the *OsINO1* and the *PcINO1* genes in the transformed cells grown either in absence of NaCl or in presence 400 mM NaCl was analysed by SDS-PAGE (Fig. 1C) and confirmed by corresponding Western blot analysis using polyclonal anti-OsMIPS antibody (Fig. 1D) indicating uniform expression of the transgene(s) in all the transformants. The MIPS proteins isolated from the *OsINO1* and *PcINO1* transformed *E. coli* cells retained their characteristic *in vitro* salt-sensitivity and salt-tolerance character, respectively (Fig. 1E).

Functional introgression of the *OsINO1* and the *PcINO1* resulted in cellular production of inositol in the transformed *E. coli* (Fig. 1F). In absence of salt, both the *OsINO1* and *PcINO1* transformed cells produced almost equal amount of free inositol. However, in presence of increasing NaCl, progressive decrease in the inositol content was noted in

OsINO1 transformed cells while the *PcINO1* transformed cells continued production of equal or about 75% of inositol when compared with the system without NaCl and 3–5 times more than the *OsINO1* transformed system. Some amount of inositol, albeit low, was detected in the empty vector transformed cells, presumably resulting from cellular transport.

Because the above experiments suggest a possible osmoprotective role of inositol, attempts were made to evaluate the growth on solid LB media of untransformed *E. coli* cells under high salt condition in presence and absence of externally supplied inositol. Preliminary experiments suggested lack of any effect of added inositol (100 mM) on normal growth of untransformed *E. coli* BL21(DE3) cells under the experimental conditions (Fig. 1G, lanes a and b). Untransformed *E. coli* BL21(DE3) cells plated on media containing 400 mM NaCl alone failed to show any growth after 16 h of incubation at 37 °C (Fig. 1G, lane c). In contrast, a gradual increase in the protection of salt induced growth inhibition was observed when cells were grown in media containing 400 mM NaCl and 10–100 mM inositol, highest protection being observed in 80–100 mM inositol (Fig. 1G, lanes d–i).

In a similar fashion, *S. pombe* cells, transformed with *OsINO1* and *PcINO1* along with empty vector constructs were allowed to grow on EMM plates with increasing NaCl concentration. Three independent groups of transformed cell lines were used for experimentation. When transformed *S. pombe* cells were exposed to saline environment, *PcINO1* transformed cells continue to grow over a range of increasing NaCl concentration and upto 400–500 mM NaCl, albeit to a lesser extent, whereas *OsINO1* transformed or empty vector transformed cells failed to overcome such adversity as assessed visually (Fig. 2A) or as measured in terms of viable cell count (Fig. 2B). Functional expression of the different gene products were analyzed through SDS-PAGE and immunodetection (Fig. 2C and D), showing uniform expression of the MIPS protein. The *OsINO1* and *PcINO1* gene products from the respective transformed cells exhibit the expected salt-sensitivity and salt-tolerance respectively in their functional MIPS activity in presence of NaCl (Fig. 2E). Further, the growth of the different cell types correlates well with the pattern of intracellular inositol production (Fig. 2F). The cells were grown in presence of 2 μ M inositol that accounts for the negligible inositol found in the pREP1 transformed cells. As in the case of *E. coli*, survival of the untransformed *S. pombe* cells under saline environment by inositol is also documented (Fig. 2G). Thus, admittedly salt tolerant character of *PcINO1* gene/gene product enhances the chances of survival of salt-stressed cell even in this unicellular eukaryote.

Inositol has been shown to accumulate in higher plants which supposedly act as an osmolyte in addition to its' other metabolic roles [32]. Although all plants harbor the *INO1* gene, the MIPS coded by most of them are known to be salt sensitive and thus whenever salt creates an osmotic imbalance within the cell this protective osmolyte cannot be synthesized in sufficient amounts. It was thought worthwhile to investigate as to whether upon introgression, *PcINO1* retains its salt-tolerant property and confers increased degree of salt-tolerance to the crop plants similar to tobacco plants demonstrated earlier [14]. To determine this, the *PcINO1* gene was cloned in a suitable plant expression vector and introgressed in indica rice, *Oryza sativa* (variety Pusa Basmati 1) and *Brassica juncea*

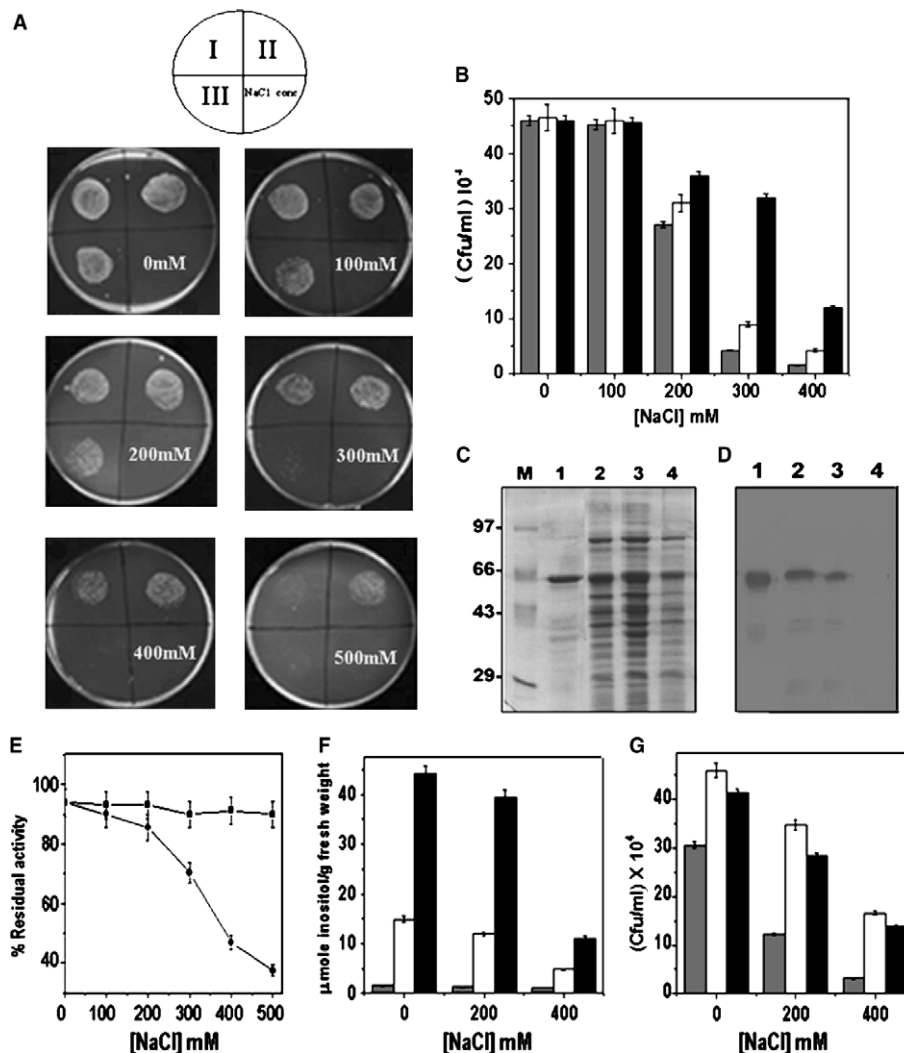


Fig. 2. (A) Growth of *OsINO1*-I, *PcINO1*-II and pREP1-III transformed *S. pombe* in solid media in presence of increasing NaCl concentration. (B) Bar graph showing the number of viable cells forming colonies in presence of different NaCl concentration. Cell survival is expressed in colony forming units (Cfu)/ml; pREP1 transformed *S. pombe* cells (grey bar); *OsINO1* transformed *S. pombe* cells (white bar) and *PcINO1* transformed *S. pombe* cells (black bar). (C and D) 10% SDS-PAGE (C) and corresponding Western blot analysis using anti Os-MIPS antibody (D) of total cellular protein extract from *S. pombe* cells transformed with *PcINO1* (lane 2), *OsINO1* (lane 3) and pREP1 (lane 4). Lane M – MW markers; lane 1- partially purified *OsINO1* protein. (E) Enzyme activity assay of purified MIPS isolated from *OsINO1* (●) and *PcINO1* (■) transformed *S. pombe* cells in presence of NaCl. Error bars indicate standard deviation from three independent determinations. (F) Free *myo*-inositol content in μmole/gm of fresh weight of the transformed *S. pombe* cell both under salt stressed and unstressed condition. Grey bar represents pREP1 transformed cells; White bar represents *OsINO1* transformed cells and black bar represents *PcINO1* transformed cells. Error bars indicate standard deviation from three independent estimations. (G) Bar graph showing the growth pattern of *S. pombe* PR109 in solid EMM media in presence of increasing NaCl concentration supplemented with different amount of inositol. Cell survival is expressed in colony forming units (Cfu)/ml of *S. pombe* cells aided with 20 μM (grey bar), 50 μM (white bar) and 75 μM (black bar) inositol. In all cases, three independently transformed *OsINO1*, *PcINO1* and pREP1 transformed *Schizosaccharomyces pombe* PR109 cell lines grown in EMM media supplemented with 2 μM inositol at 30 °C were used.

(variety B85) as detailed in Section 2.5 along with *OsINO1* as a positive control. Independent T₀ *PcINO1* and *OsINO1* transformants and untransformed control *Oryza* and *Brassica* plants were monitored for their growth in increasing NaCl concentrations (Fig. 3A and 4A). The transformed plants were checked for introgression of *OsINO1*, *PcINO1* and *hpt* gene(s) by PCR amplification (Figs. 3B–D and 4B–D) and the introgression of the *PcINO1* gene was also confirmed by southern hybridization (Figs. 3E and 4E). MIPS proteins purified from the control, *OsINO1* and *PcINO1* transformed *Oryza* and *Brassica* plants showed the characteristic salt-sensitivity and -tolerance when assayed *in vitro* (Figs. 3F and 4F) adding

further evidence of functional introgression of the corresponding gene(s).

Increase in NaCl concentration causes pronounced growth inhibition and chlorophyll loss in control and *OsINO1* transformed plants whilst *PcINO1* transformed plants showed continued, albeit less vigorous, growth upto 200 mM NaCl at least for 21 days (Fig. 3A) as also evident in the analysis of the fresh weight of shoot (Fig. 3G) and root (Fig. 3H). Although no plants could survive a stress of 300 mM NaCl for 21 days, only the *PcINO1* transformed plants could recover to normal growth after 10 days of 300 mM NaCl stress when put back to a salt-free medium (data not shown). In case of *Brassica*

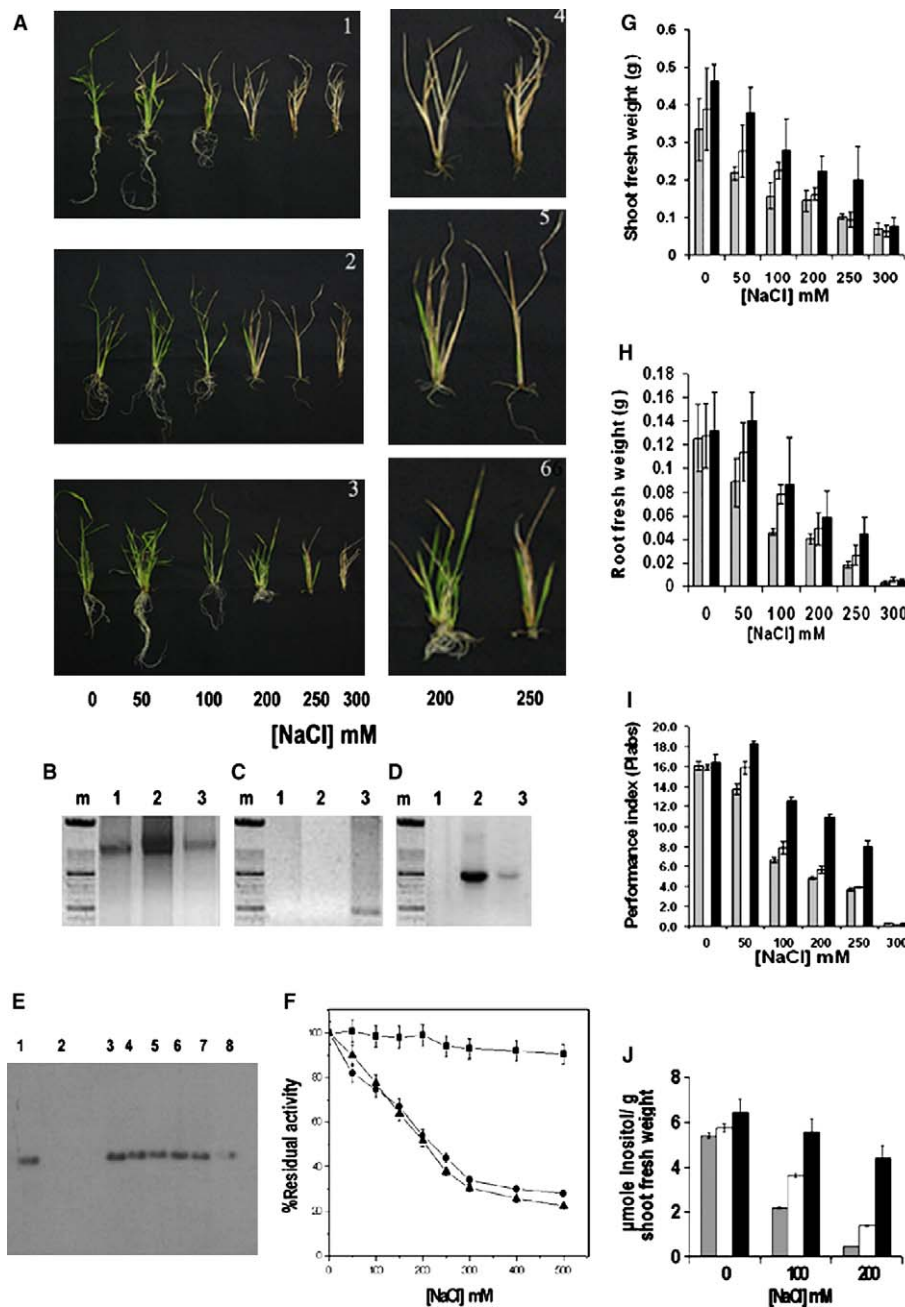


Fig. 3. (A) (1–6) Growth pattern of untransformed control (1), *OsINO1* transformed line R20 (2), and *PcINO1* transformed line P3 (3) grown in hormone free Murashige and Skoog medium with increasing NaCl concentration (0, 50, 100, 200, 250 and 300 mM NaCl) for 21 days. Enlarged view of untransformed control, *OsINO1* transformed and *PcINO1* transformed plants grown in 200 and 250 mM NaCl are shown in 4–6, respectively. (B)–(D) Representative PCR analysis of untransformed control (lane 1), *OsINO1* (lane 2, line R5) and *PcINO1*-transformed (lane 3, line P3) *Oryza* plants with *OsINO1* gene specific primers (B); with 430 bp *PcINO1* specific primers (C) and with *hpt* specific primers (D). m represents DNA marker. (E) Southern blot hybridization of *OsINO1* and *PcINO1* transgenic lines with the genomic DNA digested with *EcoRI* using *PcINO1* specific 430 bp region as the probe. Lane 1: positive control, lane 2: *OsINO1* transformed plant (line R5) and lanes 3–8: six *PcINO1* transformed lines (P3, P8, P9, P10, P12 and P20). (F) Activity of MIPS protein purified from untransformed control (▲), *OsINO1* (●) (R9, R19 and R20 lines) and *PcINO1* (■) (P3, P20 and P22 lines) transformed Pusa Basmati 1 in increasing NaCl concentration. The error bars show average deviation of the MIPS specific activity values from different lines. (G and H) Fresh weight of shoot (gm) (G) and fresh weight of root (gm) (H) of untransformed control (grey), *OsINO1* (8 T₀ lines – R5, R6, R7, R9, R12, R16, R19 and R20) (white) and *PcINO1* (7 T₀ lines – P3, P8, P9, P10, P12, P20 and P22) (black) transformed lines grown in MS medium in presence of 0, 50, 100, 200, 250 and 300 mM NaCl for 21 days. The average values of different independent lines are plotted. The error bars represent average deviation of values of independent control and transformed lines. (I) Photosynthetic efficiency or vitality index of untransformed control (grey), *OsINO1* (white) and *PcINO1* (black) transformed plants grown in presence of increasing NaCl concentration for 21 days measured by JIP test formulae from chlorophyll ‘a’ fluorescence. Average photosynthetic efficiency values of individual transgenic plants (7 T₀ *PcINO1* transformed lines P3, P8, P9, P10, P12, P20 and P22 and 8 T₀ *OsINO1* transformed lines R5, R6, R7, R9, R12, R16, R19 and R20) from each different transgenic lines were plotted. The error bars show the average deviation of photosynthetic efficiencies. (J) Shoot *myo*-inositol content in $\mu\text{mole/gm}$ fresh weight of untransformed control (grey), *OsINO1* (white, lines R5, R6, R7, R9, R12, R16) and *PcINO1* (black, lines P3, P8, P9, P10, P12, P20) transformed *Oryza* grown in increasing NaCl concentration assayed by GLC. The error bars represent average deviation of values of independent control and transformed lines.

also, the salt induced chlorophyll loss and decreased root growth is clearly visible in control and *OsINO1* transformed plants at 200 mM (the effect being more pronounced at 300 mM NaCl). On the contrary, *PcINO1* transformed plants showed much better growth under similar condition (Fig. 4A). Such growth pattern is also illustrated by the shoot (Fig. 4G)

and root fresh weight (Fig. 4H) in different concentrations of NaCl. The *PcINO1* transformed plants also recover to a normal growth from 300 mM NaCl stress for 10 days in contrast to the other two types (data not shown). At 400 mM NaCl, *PcINO1* transformed plants although remained greener than the other two, showed stunted growth and in due course, died.

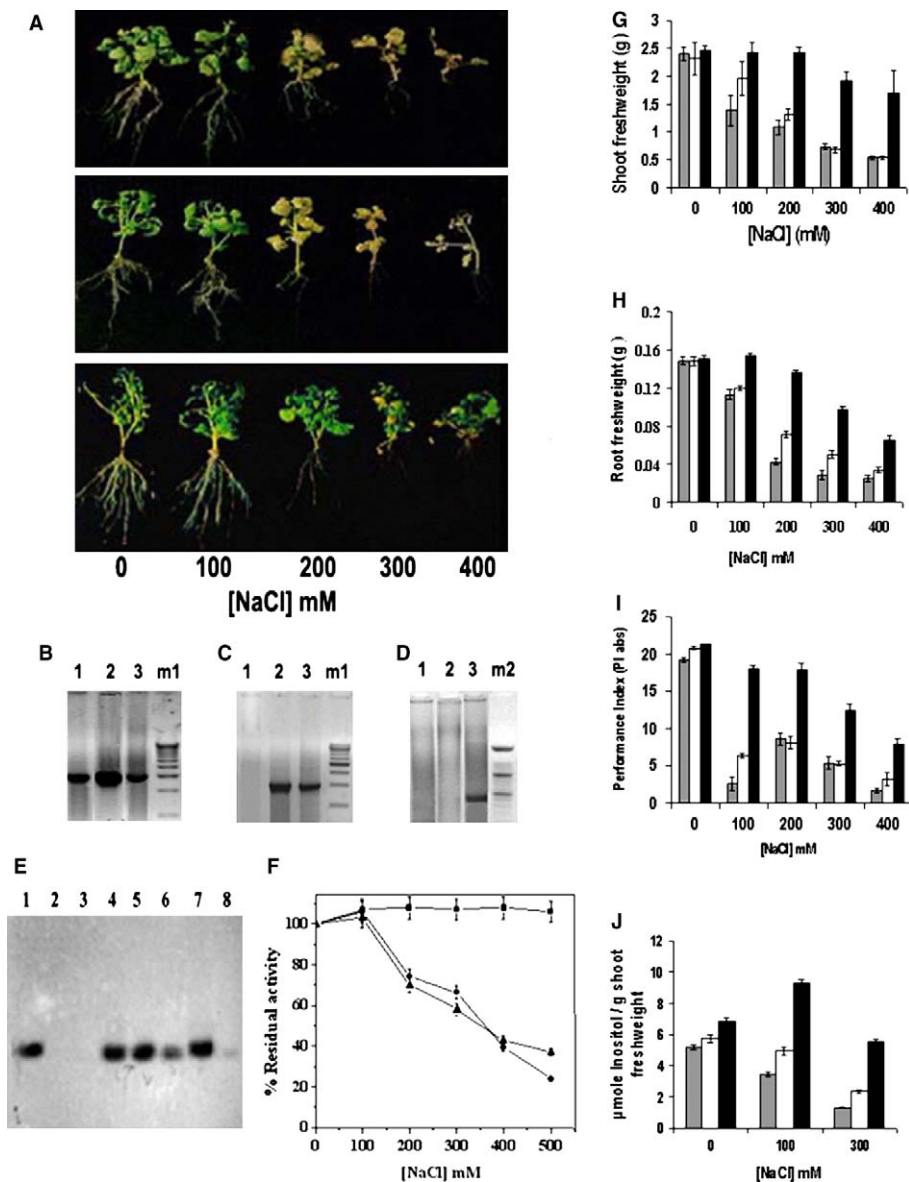


Fig. 4. A. Growth of untransformed control (uppermost panel), *OsINO1* transformed (middle panel, line Rb2), and *PcINO1* transformed (lower panel, line Pb4) *B. juncea* in different NaCl concentrations (mM). (B)–(D) Representative figure of PCR analysis of the untransformed control (lane 1), *OsINO1* (lane 2, line Rb2) and *PcINO1* transformed (lane 3, line Pb4) *B. juncea*: with *OsINO1* primers (B), with *hptII* specific primers (C) and with *PcINO1* specific primers (D). m1 represents 500 bp ladder and m2 represents 100 bp ladder. (E) Southern blot hybridisation of the transgenic lines with the genomic DNA digested with *EcoRI*; lane 1: positive control, lane 2: *OsINO1* transformed line (Rb2) and lanes 3–7: five *PcINO1* transformed lines (Pb2, Pb4, Pb9, Pb11, and Pb19) using *PcINO1* specific 430 bp region as the probe. (F) Effect of increasing NaCl concentration on enzyme activity of cytosolic MIPS isolated from control (▲), *OsINO1* (●) and *PINO1* (■) transformed *B. juncea*. The figure shows the average of three different independent transgenic lines (Pb2, Pb4, Pb11, Rb2, Rb15 and Rb19) and the bars show deviation from the mean. (G and H) Fresh weight of shoot (G) and fresh weight of root (H) of untransformed control (Grey bar), *OsINO1* (6 T₀ lines-Rb2, Rb6, Rb12, Rb15, Rb19 and Rb25, white bar) and *PcINO1* (6 T₀ lines- Pb2, Pb4, Pb9, Pb11, Pb19, and Pb21, black bar) transformed *B. juncea* in different concentrations of NaCl after 10 days. The error bars represent average deviation of values of independent control and transformed lines. (I) Average photosynthetic efficiency values measured in terms of performance index [PI(ABS)] of Control (grey bar) and 6 individual transgenic plants of *OsINO1* (white bar, Rb2, Rb6, Rb12, Rb15, Rb19, and Rb25) and *PcINO1* (black bar, Pb2, Pb4, Pb9, Pb11, Pb19 and Pb21) plants. The error bars show the average deviation of photosynthetic efficiencies. (J) Shoot *myo*-inositol content expressed as μmole/g of fresh weight of control (grey bar), *OsINO1* transformed (white bar, lines Rb2, Rb6, Rb12, Rb15, Rb19 and Rb25) and *PcINO1* (black bar, lines Pb2, Pb4, Pb9, Pb11, Pb19 and Pb21) transformed *B. juncea* in different NaCl concentrations, analysed through GLC. The error bars represent the mean deviation.

These observations validate the fact that the *PcINO1* transformed crop plants do show a better survival under salinity stress. Although cytoplasmic NaCl concentrations higher than 100 mM are unlikely to occur, the salt-tolerance property of the *Pc-MIPS* may be of relevance under conditions of higher salt accumulation.

It is known that photosynthesis and the electron transport system are adversely affected under stress [33,34]. Hence to assess the role of increased inositol production in *PcINO1* transformed plants on amelioration of salt-effects on photosynthesis, photosynthetic efficiency of the plants grown in increasing salt were measured as performance index (PI abs) at equal absorption scale from 'chlorophyll *a*' fluorescence of PS II using JIP test formulae [28]. At 200 and 250 mM NaCl, *PcINO1* transformed rice plants show more than ~2–3-fold higher photosynthetic efficiency over the other two (Fig. 3 I). *Brassica* plants also show an appreciable ~2–4-fold better photosynthetic performance for *PcINO1* transformants at 100, 200 or 300 mM NaCl than the other two (Fig. 4 I). These results indicate a substantial protection of photosystems especially PSII in *PcINO1* transgenic plants and the presumptive role of *myo*-inositol in protecting the chloroplast functions from oxidative damage resulting in better growth performance of *PcINO1* transformed plants under saline conditions.

Analysis of the total *myo*-inositol content of the transformed systems and the untransformed control plants under NaCl growth (Fig. 3J and 4J) point towards a probable correlation between the photosynthetic efficiency and the cyclitol content. *PcINO1* transformed rice and *Brassica* plants produced comparable amount of inositol at non-saline conditions. A significant reduction to 40% for control and 65% for *OsINO1* plants at 100 mM NaCl and a drastic reduction to 10% for control and 25% for *OsINO1* plants in 200 mM was observed in rice. Under similar conditions *PcINO1* transformed rice plants produced almost 95% and 80% of inositol as compared to no salt conditions. In *Brassica*, however, it was observed that in lower concentrations of NaCl i.e. 100 mM, the control plants could maintain upto 66% of the total *myo*-inositol content and the *OsINO1* transformed plants were competent to maintain 85% of the inositol pool. In comparison to these, the *PcINO1* transformed plants were observed to have rather an increased amount of total inositol level in 100 mM NaCl concentration to 135% in contrast to the same under non-saline conditions. The correlation between increased level of *myo*-inositol and salt tolerance of the *PcINO1* transformed plants makes it evident that like *E. coli* and *S. pombe*, the increased cellular level of inositol due to its unabated synthesis is primarily responsible for the protection of the system against salinity stress.

4. Conclusion

The experimental evidence presented herein confirm that the unique salt tolerant MIPS coding gene, *PcINO1*, confers salt tolerance to various levels to a prokaryote, an eukaryote and also to higher plants. The results suggest that unabated production of inositol by the *PcINO1* gene indeed is responsible for survival of the NaCl stressed cells. The present work demonstrates that *PcINO1* can singly perform a function commonly known to be regulated by a number of genes.

No single gene has so far been shown to confer salt tolerance with the exception of *AtNHX1* [11] and to such diverse biological systems with the limited exception of glycine betaine overproduction [5,35] or the mangrove allene oxide cyclase [36] and a serine-rich protein from *Porteresia coarctata* [37] affecting salt tolerance to prokaryotes and eukaryotes. Inositol or the intermediates of inositol biosynthesis, being innocuous chemicals, are not toxic to the cell either and overaccumulation is not detrimental to cellular functions. An evolutionary conserved pathway having the central metabolite glucose-6-phosphate as substrate for the reaction [12], metabolic engineering of inositol biosynthesis is unique among the osmolyte-accumulating pathways, and thus the present findings point towards the enormous prospect of raising salt tolerant organisms from diversified taxa by the introgression of the *PcINO1* gene.

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