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

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Manoj Majee‡, Susmita Maitra‡, Krishnarup Ghosh Dastidar‡, Sitakanta Pattnaik§§, Anirban Chatterjee‡, Nitai C. Hait‡‡, Kali Pada Das‡, and Arun Lahiri Majumder‡**

From ‡Plant Molecular and Cellular Genetics and the ‡Department of Chemistry, Bose Institute (Centenary Building), P-1/12, C 1 T Scheme VII M, Kolkata 700054, India

L-myo-Inositol-1-phosphate synthase (EC 5.5.1.4, MIPS), an evolutionarily conserved enzyme protein, catalyzes the synthesis of inositol, which is implicated in a number of metabolic reactions in the biological kingdom. Here we report on the isolation of the gene (PINO1) for a novel salt-tolerant MIPS from the wild halophytic rice, Porteresia coarctata (Roxb.). Tateoka. Identity of the PINO1 gene was confirmed by functional complementation in a yeast inositol auxotrophic strain. Comparison of the nucleotide and deduced amino acid sequences of PINO1 with that of the homologous gene from Oryza sativa L. (RINO1) revealed distinct differences in a stretch of 37 amino acids, between amino acids 174 and 210. Purified bacterially expressed PINO1 protein demonstrated a salt-tolerant character in vitro compared with the salt-sensitive RINO1 protein as with those purified from the native source or an expressed salt-sensitive mutant PINO1 protein wherein amino acids 174–210 have been deleted. Analysis of the salt effect on oligomerization and tryptophan fluorescence of the RINO1 and PINO1 proteins revealed that the structure of PINO1 protein is stable toward salt environment. Furthermore, introgression of PINO1 rendered transgenic tobacco plants capable of growth in 200–300 mM NaCl with retention of ~40–80% of the photosynthetic competence with concomitant increased inositol production compared with unstressed control. MIPS protein isolated from PINO1 transgenics showed salt-tolerant property in vitro confirming functional expression in planta of the PINO1 gene. To our knowledge, this is the first report of a salt-tolerant MIPS from any source.

Inositols are six-carbon cyclohexane hexitols found ubiqui-

tously in the biological kingdom, and its metabolism plays a vital role in growth regulation, membrane biogenesis, osmotol-
erance, and in many other processes. As phosphorylated deriv-
atives, its role as a phosphorus store and as a “second messen-
ger” in signal transduction pathways has long been recognized. L-myo-Inositol, physiologically the most favored stereoisomer among the eight possible geometric isomers of inositol, also enters into an array of biochemical reactions having diverse functions in cellular metabolism both as free and conjugated and phosphorylated or methylated forms (1–3).

The primary enzyme for the synthesis of 1-l-myo-inositol-1-phosphate from glucose 6-phosphate is 1-l-myo-inositol-1-phosphate synthase (EC 5.5.1.4; referred to as MIPS), which synthesizes 1-l-myo-inositol-1-phosphate through an internal oxidation-reduction reaction involving NAD+. Free inositol is generated by dephosphorylation of the MIPS product by a specific Mg2+-dependent inositol-1-phosphate phosphatase (EC 3.1.3.25). This mechanism is followed by all myo-inositol-producing organisms throughout the phylogenetic lines, and MIPS has been identified as an evolutionarily conserved protein (4). The structural gene coding for cytosolic MIPS, termed INO1, was first identified in yeast, Saccharomyces cerevisiae (5, 6) and cloned by Klig and Henry (7). Subsequently, the complete nucleotide sequence of the full-length INO1 gene from S. cerevisiae was reported by Johnson and Henry (8). Until now, over 60 genes homologous to INO1 have been cloned and characterized from a wide variety of prokaryotic, archael, and eukaryotic sources (9–12), and the conservation of a probable “core catalytic structure” among all has been proposed (12). The crystal structures of MIPS(s) from Saccharomyces and Mycobacterium have been worked out, providing evidence for the structural insight for the proposed reaction mechanism (13–17).

In addition to the cytosolic form of MIPS reported from a wide range of plant, animal, and other sources, an organellar form of the enzyme has been demonstrated in the chloroplasts of Pisum sp., Vigna radiata, Euglena gracilis, Oryza sativa, and Phaseolus sp. (18–22). The chloroplastic form of the enzyme has been found to be similar to the cytosolic enzyme, with respect to biochemical and immunological properties (20), and was demonstrated to be regulated by light and salt (21). At

1 The abbreviations used are: MIPS, L-myo-inositol-1-phosphate synthase; aa, amino acids; β-ME, β-mercaptoethanol; PMSF, phenylmethyl-

lalsufonyl fluoride; TEMED, N,N,N',N'-tetramethylethylene diamine; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; X-gl, 5-bro-

mo-4-chloro-3-indolyl β-D-galactopyranoside; Bio-ANS, bis-4,5'-8-anilino-1-

naphthalenesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
least two molecular forms of the enzyme having ~80- and ~60-kDa subunits have been identified in rice chloroplasts. Proteolytic processing of the ~80-kDa subunit to the ~60-kDa subunit followed by its phosphorylation have been identified as biochemical events resulting in activation of the chloroplastic MIPS during light and salt growth (29). Although the chloroplastic MIPS has been characterized at the enzyme protein level, identification of its structural gene is still to come. In the current section, we report the cloning and bacterial expression of the INO1 gene from Porteresia.&

To characterize the gene product with special reference to its salt tolerance property, and comparison with the O. sativa INO1 gene (RINO1) product. Further experiments report functional expression of the RINO1 and PINO1 genes as salt-sensitive and salt-tolerant MIPS proteins, respectively, upon introgression into tobacco. Introggression and expression of PINO1 allows growth of the transgenic plants in salt environment with concomitant increased inositol production and maintenance of photosynthetic potential.

EXPERIMENTAL PROCEDURES

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fuged, and both the pellet and the supernatant were analyzed by SDS-PAGE.

Solubilization and Purification of Expressed RINO1, PINO1, and ΔPINO1 Protein Products—The pellet fractions containing the expressed proteins were solubilized in a buffer containing urea (8 M urea, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM β-ME, 2 mM PMSF) and kept for 30 min at room temperature. Solubilized samples were centrifuged at 15,000 rpm for 15 min. The supernatant was dialyzed serially (step dialysis with 8, 7, 6, 5, 4, 3, and 2 M urea and finally with urea-free buffer) to remove urea. Dialyzed expressed protein sample was purified by DEAE-Sephaloc and Bio-Gel A0.5 as for the native enzyme described earlier (20).

PAGE and Western Blot Analysis—SDS-PAGE was performed according to Laemmli (30). For immunodetection, proteins were blotted onto polyvinylidene difluoride membrane, and the blot was probed with rabbit anti-MIPS antibody (1:1000) raised against purified recombinant MIPS of Entamoeba (31). Bound antibody was detected by chemiluminescence (Amersham Biosciences).

Fluorescence Spectroscopy—Tryptophan fluorescence spectra were recorded using a Hitachi F-4500 spectrofluorimeter. Protein solution (0.1 mg/ml in 20 mM Tris-HCl, pH 7.5) was taken in a quartz cuvette (4 × 4 mm). Excitation wavelength was selected at 295 nm, and the emission was scanned at a speed of 240 nm/min from 310 to 400 nm by using excitation and emission slits at 5 nm each. Each spectrum was an average of three scans. Appropriate control buffer spectra were subtracted from sample spectra to generate the fluorescence spectra of the proteins. Wavelength of maximum emission for each spectrum was determined by derivative analysis using the instrument software.

Fluorescence Quenching Experiments—Tryptophan fluorescence was quenched by titrating the protein solution with 5 mM acrylamide or potassium iodide. 2.0 ml of 0.1 mg/ml protein solution was taken in a 3-ml quartz cuvette containing a magnetic stir bar. Excitation and emission wavelengths were set at 295 and 340 nm, respectively. Freshly prepared quencher (acrylamide or potassium iodide) was added in small aliquots in the cuvette, and after each addition the solution was stirred magnetically for 1 min, and thereafter the emission reading was taken. The fluorescence readings for all concentrations of the titrant were corrected for the inner filter effect.* Due to addition of the titrant the readings were also corrected for the “inner filter effect” according to Equation 1,

\[ F_{corr} = F_{anti} \frac{A_{A} + A_{corr}}{2} \]  

where \( F \) and \( F_{corr} \) indicate the uncorrected and corrected fluorescence, and \( A_{A} \) and \( A_{corr} \) indicate the absorbance of the solution at the excitation and emission wavelengths, respectively. The quenching data were analyzed according to modified Stern-Volmer Equation 2 (32),

\[ \frac{F}{F_{0}} = 1/1 + 1/K_{stab} [Q] \]  

where \( F \) and \( F_{0} \) are the fluorescence intensities in absence and presence of the quencher; \( [Q] \) indicates the molar concentration of the quencher, and \( f_{Q} \) indicates the fraction of tryptophans accessible to the quencher. The accessible fraction \( f_{Q} \) and the effective Stern-Volmer quenching constants \( K_{stab} \) are equal to \( f_{Q} \). \( K_{stab} \) values were obtained from the ordinate intercept and slope, respectively, of the linear portion of the \( F/F_{0} \) versus \( 1/[Q] \) plot.

Bio-ANS Fluorescence Assay—Purified recombinant RINO1 and PINO1 proteins were dialyzed against 2 liters of 20 mM Tris-HCl with 10 mM β-ME. 2 ml of dialyzed protein (0.1 mg/ml) in each was taken into a 3-ml quartz cuvette containing a magnetic stirrer. Excitation and emission wavelengths were set at 370 and 490 nm, respectively. Freshly prepared Bio-ANS solution (372 μM stock solution) was added in small aliquots to the cuvette, and after each addition the solution was stirred magnetically for 1 min.

Assay for Aggregation of RINO1 and PINO1 Proteins in Vitro under NaCl Environment—Purified bacterially expressed RINO1 and PINO1 proteins (0.575 μM) were assayed for static light scattering in the absence and presence of different NaCl concentrations at 37 °C in a Shimadzu-160A spectrophotometer. Optical density of the samples at 360 nm were measured over a period of 170 min at regular intervals where increased OD was indicative of aggregation of the protein samples (33).

Construction of Plant Expression Vectors and Tobacco Transformation—Full-length clones of RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2, as obtained, were subcloned at the XbaI/KpnI site of the plant expression vector pCAMBIA1301 (a gift from Prof. Akhilesh Tyagi, University of Delhi, South Campus) under the control of the constitutive cauliflower mosaic virus 35S promoter and nopaline synthase (NOS) terminator. The vector contains the hpt gene for hygromycin resistance and gus as the reporter gene. The resultant constructs SMSA9, SMSA10, SMSMA1, and SMSMA2 contain RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 genes respectively. All the constructs and the control plasmid pCAMBIA1301 without any insert were introduced into Agrobacterium tumefaciens LBA 4404 by the freeze-thaw method (34). Tobacco leaf discs were infected with A. tumefaciens containing SMSA9, SMSA10, SMSMA1, and SMSMA2. The control plasmid pCAMBIA1301 without any insert. After 3 days of co-cultivation the leaf discs were transferred to the regeneration medium supplemented with cefotaxim (250 mg/liter) and hygromycin (15 mg/liter). Cultures were maintained at 26 °C and under continuous illumination provided by white fluorescent tubes. Shoot bud differentiation started after 14–16 days of culture. Individual shoots, which elongated into shootlets within 30–35 days. Shoots regenerated in the selection medium were transferred to hormone-free MS medium containing hygromycin (30 mg/liter) and cefotaxim (250 mg/liter) and allowed to develop roots in this medium. Ten putative transformed plants for each construct were screened for stable integration of gus, RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 genes. For histochemical gus assay, leaf segments and roots taken from the putative transformants (T0) were incubated in a buffer containing 100 mM phosphate, 0.5 mM disodium EDTA, 0.5 mM ferro- and ferricyanide, and 0.1% X-glu dissolved in dimethylformamide at 37 °C for 10–12 h (35). For analysis of introgression of RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 genes by PCR, genomic DNA was isolated (36) from young leaves of both the transformed and untransformed T0 plants, and PCR amplification of RINO1 and PINO1 genes were done following the conditions mentioned earlier. The transgenic T0 plants were allowed to flower and set seeds by preventing cross-pollination. Seeds were collected from transformed plants and germinated on medium containing hygromycin (20 mg/liter). Seedlings (T1) resistant to hygromycin were maintained to full growth at 25 ± 2 °C and a 16-h light and 8-h dark cycle in the culture room. Plants were analyzed for their photosynthetic efficiency by the Photosynthetic Efficiency Analyzer (Handy-PEA, Hansatech, UK). Photosynthetic performance of individual plants was calculated following the method of Strasser et al. (37). The analysis was done with the kind cooperation from Dr. A. K. Mishra, Utkal University, Bhubaneswar, India.

Estimation of Inositol Content of Transgenic Plants—For isolation and estimation of inositol content of the transgenic plants, the method of Bielecki and Redgwell (38) was followed. Leaf tissue (~500 mg) was homogenized with a mixture of methanol/chloroform/water/trichloroacetic acid (12:5:2:1) for isolation of total water-soluble sugars and polys. Chloroform and water (1:1) was added to the homogenized sample for phase separation. The aqueous phase containing the bulk of the water soluble plant metabolites including inositol was lyophilized to complete dryness.

For gas chromatography analysis, the lyophilized samples were transferred quantitatively to glass reaction vials in pyridine and evaporated under high vacuum. All the samples were kept in a vacuum for 24 h over P2O5 for complete removal of pyridine and water. The remaining samples were tetramethylsilane-derivatized (39) with Tri-Sil-Z (Pierce) and were run through gas-liquid chromatography in a Chemito 1000 gas chromatograph equipped with flame ionization detector. Gas chromatography conditions are as follows: 3% SP-2100 stationary phase (Supelco) supported on chromosorb-W (Sigma) packed in a 1.8-m (length) × 2-mm (inner diameter) glass column with N2 (flow rate 31 ml/min) as carrier gas, and the oven temperature programmed between 130 and 320 °C at 10 °C/min. Quantification was made against runs with authentic myo-inositol as standard.

RESULTS

Cloning and Sequencing of the Porteresia Gene (PIN01) for MIPS and Its Comparison with That from Oryza (RINO1)—Cloning and sequencing of RINO1 and PINO1 genes have been described under “Experimental Procedures,” and the complete nucleotide sequences of PIN01 are presented in Fig. 1A. Comparison of deduced amino acid sequences of PIN01 with those of RINO1 (Fig. 1B) revealed that they are considerably non-identical in which the RINO1 and PIN01 differ in the amino acid sequences for a stretch of about 147 amino acids in the mid-portion (between amino acids 173 and 320 of PIN01); the other parts of the gene bear complete identity with RINO1. More specifically, several additional and deletions of amino acids between RINO1 and PIN01 are noteworthy. These are as
follows: additions of other amino acids in PINO1 at positions 174–180 (WCLSLAS), 185/186 (SS), 239 (Cys), 273 (Ser), and 304 (Pro), whereas deletions of RINO1 amino acids in PINO1 at positions 184–186 (DVI), 195–200 (NNVIKG), and 245 (Asp). Such alterations make the PINO1 gene product as composed of a total of 512 amino acids, longer than that of RINO1 by 2 amino acids.

Functional Complementation of PINO1 in Yeast—For functional identification of the PINO1 gene, a complementation experiment was performed in which a yeast inositol auxotrophic strain, FY250, was used. The PINO1 PCR product was cloned into the yeast multicopy p426 GAPDH vector and transformed into the yeast strain FY250, along with appropriate controls as described under “Experimental Procedures.” The FY250 strains, transformed with PINO1, RINO1 (as a positive control), or control vectors without any insert were grown in the presence or absence of inositol. Results presented in Fig. 2 show that although all three transformed FY250 strains grew well in presence of 10 μM inositol and without uracil (Fig. 2, left panel), only the FY250 strains transformed with p426 GAPDH having either RINO1 or PINO1 grew in the absence of both inositol and uracil (Fig. 2, right panel), providing evidence for functional complementation of inositol biosynthesis by PINO1, as with RINO1, in the auxotrophic yeast strain. The mutant ΔPINO1-2 was similarly identified as complementing the inositol auxotroph, whereas ΔPINO1-1 failed to do so (data not presented).

Bacterial Overexpression and Purification of the RINO1 and PINO1 Proteins—In order to characterize the purified gene products of PINO1 and RINO1, the corresponding PCR products were cloned into the bacterial expression vector pET-20b(+), and induction of the gene products by IPTG was achieved as described under “Experimental Procedures.” Both RINO1 and PINO1 were expressed as the expected ~60-kDa

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**Relevant Figure:**

**Fig. 1.** A, complete nucleotide sequences and the deduced amino acid sequence of PINO1 (GenBank™ accession number AF412340). B, comparison of deduced amino acid sequences of RINO1 (GenBank™ accession number AB012107) and PINO1 through BLAST analysis.
proteins predominantly in the particulate fraction as judged by SDS-PAGE of the induced cells. The particulate material was solubilized in 8 M urea buffer and further analyzed. The expressed RINO1 and PINO1 proteins were recovered in the soluble fraction both on SDS-PAGE and the corresponding Western blots (Fig. 3, A and B).

The expressed RINO1 and PINO1 proteins were purified to homogeneity following the procedure of DEAE-Sephadex chromatography and Bio-Gel A0.5 filtration as outlined under “Experimental Procedures.” Results presented in Fig. 3 (C and D) show elution of both RINO1 (C) and PINO1 (D) proteins as near-symmetric protein peaks coincident with the MIPS activity, suggesting homogeneity of the purified proteins. Active fractions of the individual peaks show single protein bands on SDS-PAGE (Fig. 3, C and D, inset, left panel) and subsequent Western blots (Fig. 3, C and D, inset, right panel). The ΔPINO1-1 and ΔPINO1-2 gene(s) were similarly expressed, and the protein was purified to homogeneity through Western blot analysis and enzyme assay, respectively (data not presented).

Biochemical Characterization of the Expressed RINO1 and PINO1 Gene Products—Both the purified and expressed RINO1 and PINO1 proteins were biochemically characterized and compared with the corresponding enzyme proteins isolated from native sources (Table I). Estimates of $K_m$ and $V_{max}$ values for the substrate (Glc-6-P) and co-factor (NAD) were obtained with Bio-Gel A0.5 purified proteins using the Lineweaver-Burk plot. The purified recombinant RINO1 and PINO1 proteins show higher $K_m$ values for Glc-6-P than that for the corresponding purified native enzymes. The lower $K_m$ values for Glc-6-P...
for recombinant PINO1 protein suggest a higher substrate specificity compared with the recombinant RINO1 proteins. For both the proteins, the optimum temperature for enzyme activity was at 37 °C, and at optimum pH for RINO1 and PINO1 recombinant proteins the activities were between 7.5 and 8.

The expressed RINO1 and the PINO1 proteins or the corresponding purified native MIPS enzymes differed greatly in their response to in vitro NaCl concentration (Fig. 4, A and B). As evident, both the native and the expressed RINO1 proteins exhibit concentration-dependent inhibition of the enzyme activity in vitro in the presence of increasing NaCl concentrations, the MIPS activity being completely abolished at 500 mM NaCl. In striking contrast to such situations, both the native and the expressed PINO1 proteins showed no inhibition of enzyme activity in vitro up to a concentration of 500 mM NaCl. Most interesting, although the bacterially expressed $H_{9004}$ PINO1-2 gene product was catalytically active as the PINO1 gene product, it shows inhibition of the enzyme activity in the presence of increasing NaCl concentrations as in case of the RINO1 protein (Fig. 4B). The $\Delta$PINO1-1 gene product, on the other hand, although immunologically cross-reactive to the same anti-MIPS antibody, was catalytically inactive (data not presented).

Structural Basis of Salt Tolerance of the PINO1 Protein as Compared with the Salt-sensitive RINO1 Protein—In order to understand the nature of the structural changes in RINO1 protein causing in vitro inhibition of enzymatic activity due to addition of salt in contrast to the PINO1 protein (Fig. 4, A and B), we performed gel permeation chromatography on Superose 12 of RINO1 and PINO1 proteins both in the presence and

![Fig. 3. Bacterial expression, solubilization, and purification of RINO1 and PINO1 recombinant proteins. A, 10% SDS-PAGE of solubilized overexpressed RINO1 and PINO1 protein. Lane 1, solubilized pellet (RINO1). Lane 2, solubilized supernatant (RINO1). Lane 3 solubilized pellet (PINO1). Lane 4, solubilized supernatant. Molecular weights of the SDS-PAGE markers are shown at the extreme left lane. B, Western blot analysis of corresponding SDS-PAGE (A) hybridized with E. histolytica MIPS antibody (1:1000 dilution) and visualized by chemiluminescence. C, purification of RINO1 recombinant protein by Bio-Gel A0.5 gel filtration. Left side inset shows 10% SDS-PAGE of Bio-Gel A0.5 fractions. Right side inset shows Western blot of corresponding SDS-PAGE hybridized with E. histolytica MIPS antibody (1:1000 dilution) and visualized by chemiluminescence. Δ, MIPS; G, phosphatase; A$_{280}$. D, purification of PINO1 recombinant protein by Bio-Gel A0.5 gel filtration. Left side inset shows 10% SDS-PAGE of Bio-Gel A0.5 fractions. Right side inset shows Western blot of corresponding SDS-PAGE hybridized with E. histolytica MIPS antibody (1:1000 dilution) and visualized by chemiluminescence. Δ, MIPS; G, phosphatase; A$_{280}$.

| Characters | Native | Recombinant | Native$^a$ | Recombinant |
|------------|--------|-------------|------------|-------------|
| $K_m$ Glc-6-P | 1.8 mM | 2.5 mM | 1.97 mM | 3.0 mM |
| $V_{max}$ Glc-6-P | 0.153 mM | 0.166 mM | 0.14 mM | 0.188 mM |
| $K_m$ NAD | 0.08 μmol-min | 0.095 μmol-min | 0.07 μmol-min | 0.072 μmol-min |
| $V_{max}$ NAD | 0.12 μmol/min | 0.087 μmol/min | 0.09 μmol/min | 0.068 μmol/min |
| pH optimum | 7.5 | 8.0 | 8.2 | 7.5 |
| Temperature optimum | 35 °C | 37 °C | 35 °C | 37 °C |
| Molecular mass | ~180 kDa | ~180 kDa | ~180 kDa | ~180 kDa |
| Subunit | ~60 kDa | ~60 kDa | ~60 kDa | ~60 kDa |

$^a$ Data are from Ref. 20.
This experiment suggests oligomerization of the RINO1 protein blots of different fractions (Fig. 5, C). Protein was further verified in experiments described in Fig. 400 mM NaCl and without any change in enzymatic activity (Fig. 5). RINO1 elutes at the same place as a native protein in the absence of added salt. Results of such experiments are shown in Fig. 5 (A–D). It is seen that whereas the RINO1 protein is eluted as a single peak in the absence of salt (Fig. 5A), addition of 400 mM NaCl during chromatography leads to reduction in the original enzyme activity peak with concurrent appearance of high molecular weight enzymatically inactive fractions (Fig. 5B). The change in the enzyme elution pattern was judged by enzyme assay and detection of the MIPS protein by SDS-PAGE (Fig. 5, A and B, inset, left panel) and corresponding immunoblots of different fractions (Fig. 5, A and B, inset, right panel). This experiment suggests oligomerization of the RINO1 protein in the presence of 400 mM NaCl resulting in considerable decline in enzyme activity. In contrast to such situations, the PINO1 protein elutes at the same place as a native protein in the gel filtration column both in the presence and absence of 400 mM NaCl and without any change in enzymatic activity (Fig. 5, C and D). The salt-induced oligomerization of RINO1 protein was further verified in experiments described in Fig. 5E, where increasing aggregation of RINO1 protein in vitro at 37 °C was noted with increasing NaCl concentration. In contrast, the PINO1 protein did not show any aggregation with increasing NaCl concentrations under identical conditions (Fig. 5F).

Next, the tryptophan fluorescence spectra of the recombinant RINO1 and PINO1 proteins under different conditions were investigated (Fig. 6, A–E). In the absence of added salt, RINO1 protein shows significantly higher tryptophan fluorescence intensity than the PINO1 protein at the wavelength of maximum emission (336 nm). Tryptophan fluorescence intensity of RINO1 is quenched significantly in the presence of increasing NaCl concentrations, whereas that of PINO1 remains rather unaltered. It is also interesting to note that at a salt concentration of over 600 mM, the fluorescence intensities of both RINO1 and PINO1 become comparable (Fig. 6, A and B).

To understand further the differential behavior of RINO1 and PINO1 toward NaCl, we carried out tryptophan fluorescence quenching experiments where acrylamide and iodide were used as the complementary set of water-soluble quenchers. Acrylamide is a neutral quencher and is known to have the ability to penetrate into the protein interior. On the contrary, iodide is a negatively charged and highly hydrated bulky quencher having no ability to penetrate the protein interior, its quenching ability being mainly dependent on the location of the neighboring charged groups. We have analyzed our data by assuming varied heterogeneous emissions from multiple tryptophans, and we report the quenching constant as the effective Stern-Volmer constant ($K_{SV}$) that represents the weighted average of the quenching constants of the individual tryptophan residues and may contain contributions from both static and dynamic quenching. Fig. 6C shows the acrylamide quenching data in the form of the modified Stern-Volmer plot. The quenching constant ($K_{SV}$) and the quenchable fractions $f_q$ are reported in Table II. Analysis of such data shows that the RINO1 protein in absence of added salt has a ($K_{SV}$) of 6.0 M$^{-1}$ for acrylamide quenching. In the presence of 400 mM NaCl the ($K_{SV}$) of RINO1 decreased to 4.2. The ($K_{SV}$) of PINO1 remained constant around 3.4 M$^{-1}$ both in the presence and the absence of added salt. Because the microenvironment of tryptophan residues of RINO1 and PINO1 proteins is very similar, the changes in the quenching constants in the absence of salt reflect changes in the outer surface of the proteins making penetration of acrylamide relatively easier in RINO1 than in PINO1. Additions of salts rearranges groups on the surface of RINO1 in such a way as to cause greater hindrance toward the penetration of acrylamide. For both RINO1 and PINO1, tryptophan fluorescence is 100% quenched by acrylamide.

The data for the quenching of tryptophan fluorescence by iodide is shown in Fig. 6D. Iodide is able to quench only about 20% of tryptophan fluorescence of PINO1 at a ($K_{SV}$) of 1.7 M$^{-1}$, and 80% of the tryptophan is not quenchable by iodide. On the contrary, about 50% of total tryptophan fluorescence of RINO1 is quenched by iodide. The quenchable groups of RINO1 are of two types. About 10% of total fluorescence is quenched with a ($K_{SV}$) of 22 M$^{-1}$, whereas 40% is quenched with a ($K_{SV}$) of 1.9 M$^{-1}$, a value very similar to that of PINO1 (Table II). Thus RINO1 has more tryptophan groups close to the surface compared with PINO1. The iodide quenching data thus reveal that although the protein interior in both RINO1 and PINO1 remains similar to each other, there is considerable difference in tryptophan accessibility due to the difference in exposition of ionic groups on the surface of the two proteins.

The differential conformational behavior of RINO1 and PINO1 proteins with added salts is also reflected in their surface hydrophobicity characteristics. Bis-ANS is a highly
conformation-sensitive fluorescent probe that binds to the exposed hydrophobic sites on the protein surfaces. In the absence of salt, RINO1 shows higher surface hydrophobicity than PINO1 (Fig. 6E). In presence of 0.4 M NaCl, surface hydrophobicity of PINO1 is slightly (−15%) reduced but that of RINO1 is reduced by more than 50%. This also shows that in the presence of salt, exposed hydrophobic groups in RINO1 get buried inside the globular structure, whereas in PINO1 such changes are minimal. Data presented in Fig. 6E also reflect that there are differences in the tertiary level of organization between RINO1 and PINO1 both in the presence and absence of salts.

Functional Introgression of RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 in Tobacco and the Phenotype of the Transgenic Plants—Experiments described in Fig. 4 suggested that the

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**Fig. 5.** Salt-induced oligomerization and aggregation of RINO1 and PINO1 recombinant proteins. A and B, elution profile of RINO1 recombinant protein on Superose 12 gel filtration column in the absence of NaCl (A) and in presence of 400 mM NaCl (B). ~1 mg of purified protein was loaded onto the column. Left-side inset shows 10% SDS-PAGE of Superose 12 fractions. Right-side inset shows immuno-dot blot analysis of corresponding fractions. ■ absorbance at 280 nm; □ M(PS activity. C and D, elution profile of PINO1 recombinant protein on Superose 12 gel filtration column in absence of NaCl (C) and in presence of 400 mM NaCl (D). ~1 mg of purified protein was loaded onto the column. Left-side inset shows 10% SDS-PAGE of Superose 12 fractions. Right-side inset shows dot blot analysis of corresponding fractions. ■ absorbance at 280 nm; □ M(PS activity. E and F, aggregation assay of RINO1 protein (E) and PINO1 protein (F) in different concentrations of NaCl. ~0.875 μM purified RINO1 protein or PINO1 protein in 20 mM Tris-HCl, pH 7.5, with 10 mM β-ME was subjected to static light scattering at 360 nm in the absence and presence of increasing concentrations of NaCl (100–500 mM) at 37 °C in Shimadzu UV-160A for up to 170 min. Line 1, 0 mM NaCl; line 2, +100 mM NaCl; line 3, +200 mM NaCl; line 4, +300 mM NaCl; line 5, +400 mM NaCl; and line 6, +500 mM NaCl.
By using an excitation wavelength of 370 nm, the intensity of Bis-ANS fluorescence emission at 490 nm with 0.1 mg/ml RINO1 or PINO1 proteins displayed a simple (3hygR:1hygS) segregation pattern in potassium iodide. C, line 1, PINO1, no salt; line 2, RINO1; line 3, RINO1 no salt. D, line 1, PINO1; line 2, RINO1; inset in D shows the initial slope and intercept of the lines 1 and 2. E, binding of Bis-ANS to RINO1 and PINO1 proteins in the presence and absence of added salt. By using an excitation wavelength of 370 nm, the intensity of Bis-ANS fluorescence emission at 490 nm with 0.1 mg/ml RINO1 or PINO1 proteins in 20 mM Tris-HCl buffer of pH 7.5 containing β-ME was measured after every addition of aliquots of acrylamide or potassium iodide. C, line 1, PINO1, no salt; line 2, RINO1 + 400 mM NaCl; line 3, RINO1 no salt. D, line 1, PINO1; line 2, RINO1; inset in D shows the initial slope and intercept of the lines 1 and 2.

The results of such experiments are depicted in Fig. 7 (A–E).

Fig. 6. A and B, tryptophan fluorescence emission spectra of RINO1 (A) and PINO1 (B) using an excitation wavelength of 295 nm. Both proteins were in 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM β-ME at a concentration of 0.1 mg/ml. Line 1, protein alone; line 2, protein + 200 mM NaCl; line 3, protein + 400 mM NaCl; line 4, protein + 500 mM NaCl. C and D, modified Stern-Volmer plot for the quenching of tryptophan fluorescence of RINO1 and PINO1 proteins by acrylamide (C) and iodide (D). 0.1 mg/ml of either RINO1 or PINO1 protein in 20 mM Tris-HCl buffer, pH 7.5, containing β-ME was excited at 295 nm, and emission intensity at 340 nm was measured after every addition of aliquots of acrylamide or potassium iodide. C, line 1, PINO1, no salt; line 2, RINO1 + 400 mM NaCl; line 3, RINO1 no salt. D, line 1, PINO1; line 2, RINO1; inset in D shows the initial slope and intercept of the lines 1 and 2.

**Table II**

| Protein         | Acrylamide | Iodide |
|-----------------|------------|--------|
|                 | $K_{sv}$ | $f_c$ | $K_{sv}$ | $f_c$ |
| RINO1, no salt  | 6.0      | 1.0   | 22       | 0.1   |
| RINO1, 0.4 mM NaCl | 4.2     | 1.0   | 24       | 0.1   |
| PINO1, no salt  | 3.4      | 1.0   | 17       | 0.2   |
| PINO1, 0.4 mM NaCl  | 3.4     | 1.0   | 17       | 0.2   |

*Fig. 7A* shows growth of transformed tobacco plants in hormone-free MS media containing different concentrations of NaCl as follows: a, empty vector; b, RINO1; c, PINO1; d, ΔPINO1-1; and e, ΔPINO1-2. All the transgenic plants could grow in a comparable manner between 0 and 100 mM NaCl media. Although the growth of the empty vector-transformed control and ΔPINO1-1 plants is severely affected along with loss of chlorophyll by the further increase in NaCl concentrations in the growth media, (A, a and d), such growth inhibition and loss of chlorophyll are less pronounced in the RINO1- and ΔPINO1-2-transformed plants (A, b, and c) under similar conditions. In contrast, however, only the PINO1-transformed plants were able to grow between 200 and 300 mM NaCl in the growth media without appreciable loss of chlorophyll or growth vigor in comparison to others (Fig. 7A, c). The difference is most evident in plants grown at 300 mM NaCl, although with a less vigorous growth of the PINO1 transformed plants (Fig. 7A, a–e).

Introggression of RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 genes in the transgenic tobacco plants was verified further by PCR amplification of the genes (Fig. 7B). Transgenic lines of tobacco plants transformed with the empty vector, RINO1 (R1 and R2), PINO1 (P4 and P8), ΔPINO1-1 (M1–3 and M1–5), and ΔPINO1-2 (M2–7 and M2–9) gene constructs showed, as expected, the characteristic tobacco INO1 PCR products when amplified with the tobacco INO1-specific primers designed after the full-length tobacco INO1 gene (GenBank™ accession number AB009881) (Fig. 7B, a–c). Only the RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 transformed tobacco plants, and not the empty vector transformed plants, showed the INO1 PCR products when amplified with the RINO1 primers designed for the full-length gene; the ΔPINO1-1 and ΔPINO1-2 transformed plants showed shorter PCR products due to the internal deletion in both (Fig. 7B, d–f). Furthermore, only the PINO1 and the ΔPINO1-1 transformed plants showed the expected ~440-
FIG. 7. A, growth pattern of empty vector transformed (line 7) (a); b, RINO1 transformed (line 2); c, PINO1 transformed (line 8); d, ΔPINO1-1 (line 3); e, ΔPINO1-2 (line 9) transformed tobacco plants in media containing increasing concentrations of NaCl. 3-Week-old plants were grown in hormone-free Murasige and Skoog medium containing different concentration of NaCl (0, 100, 200, and 300 mM) supplemented with cefotaxim (250 mg/liter) and hygromycin (25 mg/liter). The last two panels show one plant from each group grown in the presence of 200 and 300 mM of NaCl. B, PCR analysis of RINO1, PINO1, ΔPINO1-1, ΔPINO1-2 transformed and vector transformed plants. Only two lines of each among 10 lines was shown here. PCR analysis with N. tabaccum INO1 primers (a–c); RINO1-specific primers (d–f); PINO1-specific primers (g–i) (see details under “Experimental Procedures”). Ms, markers (HindIII-digested λDNA). R1, RINO1 transformed plant (line 1). R2, RINO1 transformed plant (line 2). P4, PINO1 transformed plant (line 4). P8, PINO1 transformed plant (line 8). VT, empty vector transformed control plant (line 7). M1–3, ΔPINO1-1 transformed plant (line 3). M1–5, ΔPINO1-1 transformed plant (line 5). M2–7, ΔPINO1-2 transformed plant (line 7). M2–9, ΔPINO1-2 transformed plant (line 9). C, effect of increasing NaCl on MIPS activity from vector, RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 transformed tobacco plant. ~10 µg of protein was used for enzyme assay. MIPS-specific activities are expressed as a micromoles of inositol 1-phosphate released per mg per h and represent the average of 10 individual transgenic plants of different lines. The error bars show the range of measurements for each plant. D, inositol levels in vector transformed, RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 transformed plants. 3-Week-old grown plants of each transgenic plant was assayed for inositol levels by gas-liquid chromatography as described under “Experimental Procedures.” Inositol levels are expressed as µmol/g fresh weight and indicate the average of 10 individual transgenic plant from different lines. The error bars show the range of inositol content of each plant. E, photosynthetic efficiency of vector transformed, RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 transformed plants. 3-Week-old grown
growth and photosynthetic efficiency than the plants grown in normal MS medium with 0.55 mM inositol (Fig. 8A). Photosynthetic efficiency remains unchanged in all types of plants grown at 0 or 100 mM NaCl. Reduction of photosynthetic competence to ~20% or less was noticed in wild plants growing in 200–300 mM NaCl, whereas plants grown similarly but with increased inositol (2–5 mM) retain ~70–40% photosynthetic competence in comparison to the corresponding untransformed plants (Fig. 8B).

**DISCUSSION**

Although studies during the last decade have enhanced our understanding of plant responses to a stressful environment, there are still many pieces to the puzzle that elude mechanistic insights. One aspect, separate from the current emphasis on stress sensing and signaling, deals with questions about evolutionary changes of protein sequence and structure that may be adaptive to functioning under stressed conditions. Are the proteins, or at least some of them, crucial for proper functioning under stress in stress-tolerant species specifically designed to function in a cellular environment characterized by tolerance to various abiotic stress factors? In case of salt tolerance, a multigenic trait, the quest for an answer to such an intriguing question resides in a genomic and proteomic comparison of the glycophytes with their halophytic models, considerations that have prompted analysis of Arabidopsis genes with those of Thellungiella, its halophytic relative (40). A similar comparison can be presumed between *O. sativa* L., the cultivated rice, and its halophytic wild relative, *P. coarctata* (Roxb.). Tateoka, which grows abundantly in the saline coastal region, and the system can very well be used for bio-prospecting of halotolerant homologues of rice genes.

To carry out an analysis that investigates such questions has been the primary objective of the present work. We have chosen the enzyme MIPS, which is conserved across evolutionarily diverse taxa (4, 12), produces inositol, and is known to function in varied biochemical activities and also during stress in both prokaryotic and eukaryotic organisms (3, 41–45). Among the 65 INO1 genes known (12), the INO1 from *Archeoglobus fulgidus* (11) has been shown to code for a thermotolerant protein, the only stress-tolerant MIPS so far reported. The present report of a salt-tolerant MIPS from *P. coarctata* is the only one known from any eukaryotic source until now.

Analysis of the nucleotide sequences of *RIN1* and *PINO1* genes establish that the *PINO1* gene differs from the *RIN1* gene considerably with respect to its organization between amino acids 173 and 320. The organization is characterized by deletion, addition, conservative substitution, and rearrangement with two additional amino acids thereby making the *PINO1* gene longer by two amino acids as enumerated (Fig. 1B). A similar situation has been reported for the allene oxide cyclase homologue (“margrin”) from the mangrove, *Bruguiera sexangula* (44), wherein a stretch of 70 amino acid residues, different in its organization from the allene oxide cyclase from *Arabidopsis* or *Lycopersicum*, conferred salt tolerance to *E. coli*, yeast, and tobacco cells. Furthermore, whether the *PINO1* gene is the only MIPS-coding gene in *Porteresia* or a duplicated parologue of *INO1* having a different organization has yet to be resolved. Functional identification of the *PINO1* gene was made by using a yeast strain FY250, auxotrophic for inositol by means of an insertion of HIS3 in the *INO1* open reading frame (9), a method adopted by others as well (9, 45).

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plants of each transgenic plant were taken, and photosynthetic efficiency was measured in different parts (base, middle, and apex) of leaf lamina as described under “Experimental Procedures.” Average photosynthetic efficiency of 10 individual transgenic plants from different lines was plotted. The error bars show the range of photosynthetic efficiency for each plant.
Overexpression of both RINO1 and PINO1 was achieved through pET-20b (+) bacterial expression vector. The protein was expressed initially in the insoluble fraction but could be solubilized by urea followed by renaturation in its active form through slow removal of urea. Both the RINO1 and PINO1 expressed proteins were purified to apparent homogeneity by procedures established earlier (Fig. 3) along with the ΔPINO1-1 and ΔPINO1-2 expressed proteins. Biochemical characterization of the RINO1 and the PINO1 expressed enzyme proteins (Table I) reveal comparable properties with the other known MIPS proteins (4). However, one striking difference between RINO1 and PINO1 was their response toward the NaCl effect in vitro. Both native and bacterially expressed PINO1 proteins turned out to be tolerant to NaCl up to 500 mM in vitro in striking contrast to the corresponding RINO1 protein (Fig. 4 A and B). However, the expressed ΔPINO1-2 protein, the truncated PINO1 protein where the stretch between aa 174 and 210 has been deleted by in vitro mutagenesis, shows salt sensitivity, the RINO1 protein, although fully active catalytically in contrast to the ΔPINO1-1, which turned out to be catalytically inactive (Fig. 4B). Such experiments suggest that this amino acid stretch is the likely functional domain for conferring salt stability in the PINO1 protein.

To search for an explanation for the structural basis of such contrasting characteristics between the RINO1 and PINO1 proteins, several experiments were performed. Gel filtration and aggregation assays of the RINO1 and PINO1 proteins as influenced by NaCl revealed that the RINO1 protein undergoes oligomerization in the presence of NaCl with concomitant loss of enzyme activity, whereas the PINO1 protein remains unaltered under similar conditions (Fig. 5). To probe into the mechanism of such salt-mediated aggregation and loss of activity, this was followed by a series of experiments in which the effect of salt on the tryptophan fluorescence of RINO1 and PINO1 proteins was monitored (Fig. 6). Progressive decrease of fluorescence intensity of RINO1 protein with increasing salt concentration (Fig. 6A) indicates structural alterations of the salt-sensitive protein. However, the emission maximum of RINO1 remains invariant as a function of increasing salt concentration, suggesting that the tryptophan environment remains unchanged. Because tryptophan residues usually remain buried within the globular structure, the salt-induced changes do not interrupt the tryptophan microenvironment. It probably moves other protein segments closer to tryptophan to facilitate energy transfer and hence reduce intensity. The structure of PINO1 protein is stable to the addition of salts. Because salts screen electrostatic interactions, there is considerable difference in the exposition of charged residues on the outer surface of RINO1 and PINO1. Furthermore, because oligomerization would affect mainly the protein surface and not the globular interior, this helps explain the changes in the acrylamide quenching constants of RINO1 with an increase in salt concentration (Table II). The penetration of the acrylamide into the globular interior through the surface is hindered due to protein-protein interaction in the oligomer, although the microenvironment of tryptophan remained unaltered. The salt sensitivity of RINO1 and its absence in PINO1 can thus be attributed to their difference in ionic environments prevailing on their surface and as evidenced from the iodide quenching data, due also to difference in hydrophobicity close to the surface.

Some preliminary structural comparisons were done on the RINO1 and PINO1 proteins after threading the two sequences on the yeast MIPS structure worked out recently (13, 14) and available in the Protein Data Bank. The models generated for RINO1 and PINO1 were compared with the yeast MIPS homolog that showed close similarity with the yeast MIPS structure. However, when the two models for RINO1 and PINO1 proteins were compared with each other, a striking difference was observed in the helix following the region where the RINO1 and PINO1 amino acid sequences differed the most (aa 174–210). The helix is continuous in the case of RINO1 but is disrupted in PINO1 (data not presented). That this break in the helix might be responsible for the structural integrity of the multimeric PINO1 protein under salt environment is evidenced by the salt sensitivity of the ΔPINO1-2 protein (Fig. 4B). Further detailed work will be required to elucidate the structure-function relationship of the two proteins in terms of salt sensitivity vis-à-vis salt tolerance.

By having established the salt-tolerant property of PINO1, an obvious question to us was its probable functional expression in planta on introgression and analysis of the transgenic plants thus generated. Transgenic tobacco plants were raised by introgression of RINO1, PINO1, ΔPINO1-1, and ΔPINO1-2 gene(s), and their growth in the presence of increasing NaCl concentrations was compared with that of empty vector transformed plants (Fig. 7). A noteworthy observation was the growth performance of the PINO1 transformed plants that showed near-normal phenotype with marginal growth reduction between 200 and 300 mM NaCl. MIPS protein(s) isolated from the variously transformed plants confirmed their characteristic salt sensitivity (RINO1 and ΔPINO1-2) or tolerance property (PINO1) in vitro (Fig. 7C), providing evidence for functional expression in planta of the different transgenes following introgression. Moreover, the specific activity of the isolated PINO1 or RINO1 proteins corresponds to that of the bacterially expressed proteins, with the PINO1 protein being enzymatically more active. Because the inherent MIPS of the tobacco plant is a salt-sensitive one, the empty vector transformed control plants and ΔPINO1-1 (catalytically dead mutant of PINO1) produced decreased amounts of inositol in increasing NaCl in the media. The sustained production of higher levels of inositol by the PINO1 transformed plants during growth between 0 and 200 mM NaCl reflects the uninhibited functioning of the PINO1 gene product under such growth conditions. Even at 300 mM NaCl, these plants produced the highest amount of inositol in comparison to both untransformed plants under salinity stress is expected to be facilitated by external supply of inositol to the growth medium. In order to verify such a possibility, wild tobacco plants were grown in vitro under increasing NaCl concentrations containing elevated levels of inositol in the growth media resulting in similar observations, thus confirming the role of inositol in salt tolerance (Fig. 8). Increased synthesis of inositol and its transport has been shown previously to play a major role in sequestration of sodium ions and maintenance of photosynthetic competence during salt stress (42, 46). A protective role for inositol in maintaining the photosynthetic efficiency of the plants, as demonstrated previously for other osmolytes like glycine-betaine or proline (43, 47–48), may also be inferred from such results.

Metabolic engineering of various pathways for production of osmolytes including polyols during salt stress have been at-
tempted by a number of investigators (43, 47–48). Regulation of inositol pathway in relation to salt stress has also been studied earlier (21, 42). In a facultative halophyte-like Mesembryanthemum crystallinum, a coordinate transcriptional induction of INO1 and the inositol methyltransferase (IMT) gene(s) under salt environment for production of both inositol and its methylated derivative, pinitol has been documented (49). Furthermore, overexpression of MIPS coded by TUR1 cDNA from Spirodea polyrrhiza in Arabidopsis led to an increased free inositol pool (50). Because both inositol and pinitol are known to act as osmoprotectants during salinity stress, in systems lacking the IMT gene and thus unable to produce pinitol, a mechanism for increased synthesis of inositol under salt environment may be provided by a salt-tolerant MIPS. The experiments described herein increase the possibility of raising salt-tolerant plants by manipulation of the evolutionarily cons-

Fig. 8. A, effect of externally added inositol on growth pattern of NaCl-grown (0–300 mM) wild tobacco plants in hormone-free MS media having 0.55 mM (a) inositol, 2 mM inositol (b), and 5 mM inositol (c). B, photosynthetic efficiency of 3-week-old grown plants (as in A). Photosynthetic efficiency was measured in different parts (base, middle, and apex) of leaf lamina as described under “Experimental Procedures.” Average photosynthetic efficiency of 10 individual plants was plotted. The error bars show the range of photosynthetic efficiency for each plant.
served inositol pathway through introduction of a salt-tolerant PINO1 gene. Furthermore, in contrast to many other systems where metabolite limitations and growth compromise due to inositol depletion (49, 51) may impose restrictions on metabolic engineering for production of different osmolytes (47) for raising salt-tolerant plants, inositol requirements for normal growth of the plant is satisfied even during stress by the salt-tolerant MIPS in addition to its proposed function as an osmolyte as enumerated here.

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