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Identification and characterization of a novel iron deficiency and salt stress responsive transcription factor IDEF1 in *Porteresia coarctata*

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Abstract

Iron availability affects plant growth depending on soil type. Mangroves are characterized by alkaline soils in which the halophytic wild rice relative *Porteresia coarctata* thrives. Young plants of *P. coarctata* grew optimally in the presence of 150 mM NaCl in a hydroponic medium and tolerated iron deficiency and salt up to 21 d without showing any symptoms of stress. A homolog of the rice *iron deficiency responsive cis-acting element binding factor* (*IDEF1*) that functions at the base of an iron regulated network was isolated and characterized from *P. coarctata*. *PcIDEF1* had a close paralog in *P. coarctata* genome and its transcript expression was upregulated by both iron deficient conditions and salt treated conditions for up to three weeks. Sub-cellular localization study suggests nuclear targeting PcIDEF1 protein in guard cells and root tissues of tobacco. *In vitro* assays for metal binding affinity and binding PcIDEF1 to *iron deficiency responsive element 1* (*IDE1*)-like elements in the 5' flanking region of an iron regulated transporter from *P. coarctata* suggest that PcIDEF1 could potentially sense iron content in a plant cell and regulate expression of iron responsive genes containing *IDE1*-like elements in their promoter region. This study provides evidence for a possible cross-talk between iron deficiency and salt responses.

Additional key words: alkaline pH, iron transporter, NaCl, tobacco, wild rice.

Introduction

Iron is an essential micronutrient for plants and its utilization depends on both edaphic factors and biological factors. Bio-available form of iron could be limited in calcareous soils with a high pH, and crop plants growing under such conditions show iron deficiency symptoms (Marschner 1995). On the contrary, a high content of iron in waterlogged anaerobic soils could be toxic for a plant. Both iron toxicity and deficiency are observed in plants growing in coastal regions, particularly in mangroves (Holmer *et al*. 1994). However, coastal plants have evolved specific mechanisms to thrive under environmental extremities that include high or low iron availability and high concentrations of NaCl (Reef *et al*. 2010**).** Salinity and the availability of utilizable iron in coastal soils could be influenced by many additional factors that are normally absent inland (Giblin and Howarth 1984, Holmer *et al*. 1994).

Porteresia coarctata is a tetraploid wild relative of rice that grows on sandy estuarine beds of mangrove wetlands. *P. coarctata* beds are generally alkaline with a pH ranging from 7 to 8.8 and the plants can survive a wide range of salinity (Jagtap *et al*. 2006). In salt marsh ecosystems, typical of *P. coarctata* (Kanal and Short 2009), the iron cycle could be result of a dynamic interaction between tidal water movement, dissolved sulphides and iron concentrations, and sulphate reduction by rhizosphere bacteria (King *et al*. 1982, Giblin and Howarth 1984). In addition, freshwater inflows during monsoon can lower the pH due to dilution of salts and change sulphur oxidation rates (Benmoussa *et al*. 1997) leading to iron excess or deficiency.

Maintenance of iron homeostasis in plants requires

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Abbreviations: BPDS - batho-phenanthroline disulphonic acid; dI-dC - deoxyinosinic-deoxycytidylic; EMSA - electrophoretic mobility shift assay; FRO - ferric chelate reductase; GFP - green fluorescent protein; His-6 - hexa-histidine; HN - histidine-asparagine, *IDE* - iron deficiency responsive element; IMAC - immobilized metal ion affinity chromatography; MS - Murashige and Skoog; ORF - open reading frame; *PcIDEF1* - *Porteresia coarctata* iron deficiency responsive *cis*-acting element binding factor 1; *PcIRT1* - *Porteresia coarctata* iron regulated transporter 1.

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careful regulation of iron uptake systems (Walker and Connolly 2008). Plants respond to iron toxicity and deficiency by modulating expression of different gene sets (Connolly and Guerinot 2002). Oxidation of Fe^{2+} to Fe^{3+} by oxygen transported from shoots to roots through aerenchyma, or avoidance of Fe^{2+} at roots constitute iron toxicity response mechanisms in plants (Majerus *et al*. 2007). In response to iron deficiency, plants have evolved two major strategies for iron uptake from soil (Römheld and Marschner 1986). The strategy I, which is followed by non-graminaceous species, comprises three steps: *1*) iron solubilization by P-type ATPase mediated proton extrusion (Santi and Schmidt 2009), *2*) reduction of Fe^{3+} to Fe^{2+} by a plasma membrane bound ferric chelate reductase (Robinson *et al.* 1996, Brumbarova *et al.* 2015), and *3*) transport of Fe^{2+} ions across the root plasmalemma by a ferrous transporter of the "ZRT, IRT-like protein" or ZIP family (Eide *et al.* 1996, Vert *et al.* 2002). In contrast, graminaceous plants use the strategy II mechanism for iron acquisition. It involves secretion of low molecular mass organic compounds, phytosiderophores of the mugineic acid family with a high affinity towards Fe^{3+} ions *via* a Transporter of mugineic acid 1 (TOM 1) (Nozoye *et al.* 2011). Subsequently, the ferric-mugineic acid complex is transported across the root plasmalemma *via* a Yellow stripe/Yellow striple-like family transporter (Jeong and Guerinot 2009). In rice, however, due to a probable adaptation to flooded conditions where reduced iron is abundantly available due to a lesser oxygen content, a part of the strategy I mechanism is induced in addition to induction of the complete strategy II mechanism upon iron deficiency (Curie *et al*. 2009). Till now, there are two functionally active Fe^{2+} transporters, OsIRT1 and OsIRT2, reported in rice, whereas activity of ferric chelate reducatse (FRO) was found to be very low (Ishimaru *et al*. 2006).

Recent findings show a deviation from the existing strategy I / strategy II concept with the identification of iron binding compounds like coumarins and flavins in root exudates of strategy I plants *Arabidopsis thaliana* and *Medicago truncatula* (Rodriguez-Celma *et al.* 2013, Fourcroy *et al.* 2014, Grillet *et al.* 2014). On the other hand, in rice, iron binding phenolic compounds were identified in root exudates apart from phytosiderophores (Bashir *et al.* 2011, Ishimaru *et al.* 2011). Once present inside roots, Fe is redistributed to different plant parts through xylem and phloem with the help of iron chelators like citrate, nicotianamine, deoxymugineic acid, and proteins, which act as chaperones and prevent iron from accumulation and generation of reactive oxygen species *via* Fenton chemistry (Koike *et al.* 2004, Rellan-Alvanez *et al.* 2010, Conte and Walker 2011, Grillet *et al.* 2014). Eventually, iron transports to sink tissues like leaves, reproductive organs, and seeds, where it is in highest demand (Roschzttardtz *et al.* 2013, Grillet *et al.* 2014).

There are no reports on molecular mechanisms that underlie iron deficiency tolerance in the presence of sodium chloride in *P. coarctata.* However, salt tolerance mechanisms in *P. coarctata* have been explored (Flowers *et al*. 1990, Majee *et al*. 2004) owing to its relation to cultivated rice, and hence, the potential for breeding for salt tolerance. Iron deficiency responsive *cis*-acting element binding factor 1 (IDEF1) has been reported to function at the base of an iron regulatory network in rice (Kobayashi *et al*. 2007). IDEF1 triggers an early response to iron deficiency by possibly sensing the cellular content ratio of iron against other metals, thereby regulating the progression of iron deficiency in rice (Kobayashi *et al*. 2012). The present study was therefore designed to investigate its homolog in *P. coarctata* (designated as *PcIDEF1*) and study its structural and functional characteristics.

Materials and methods

Porteresia coarctata (Roxb.) Tateoka plants were collected from the Pichavaram mangrove forests, Tamil Nadu, India. The plants were grown in clay soil in a greenhouse at 30 °C during day time and 25 °C during night time under natural irradiance. Since propagation by seeds is difficult in *P. coarctata* due its recalcitrant nature (Flowers *et al*. 1990), for all the treatments, young plants emerging from the rhizome of a mature plant after a month were transferred to a half strength Murashige and Skoog (1962; MS) medium. The nutrient solution was adjusted daily to a pH of 5.7 or 7.9 with 1 M NaOH and renewed weekly. The limit of NaCl tolerance was assayed hydroponically after one month of acclimatization in the above medium that contained different concentrations of NaCl. A 16-h photoperiod with an irradiance of 80 μmol m^{-2} s^{-1} and a constant temperature of 25 °C were maintained for all the treatments. For iron deficiency treatment at pH 5.7, the plants were transferred to a half strength MS medium without iron. For the medium at pH 7.9, iron was retained as in the MS medium and only the pH was adjusted. For combined iron deficiency and salt treatment, the plants were transferred to an iron free half strength MS medium containing 100, 150, or 200 mM NaCl. All the treatments were given for a period of three weeks.

Iron accumulation in leaves and roots of the treated and control *P. coarctata* plants was estimated using inductively coupled plasma mass spectrometry at *SGS India* (http://www.sgsgroup.in).

Chlorophyll was extracted from leaf tissues in 80 % (v/v) acetone according to Arnon (1949). Chlorophyll *a* and *b* content was measured spectrophotometrically (UV-1601, Shimadzu, Japan) at 663 and 645 nm, respectively.

Assay of FRO was performed as described by Romera *et al*. (1996). In brief, roots of the differentially treated

P. coarctata plants were rinsed in double distilled water and then placed in an assay solution [0.2 mM $CaSO_4$, 5 mM MES at pH 5.5, 0.1 mM Fe(III)EDTA, 0.2 mM batho-phenanthroline disulphonic acid (BPDS)]. After incubation in the dark for 30 min, an aliquot of the assay solution was taken, and absorbance at 535 nm was measured. Concentration of Fe(II)BPDS was quantified using a coefficient of absorbance of 22.14 mM^{-1} cm^{-1}.

At the end of the treatment period, the root exudates of the plants from each treatment were collected as described by Takagi (1976). The root and leaf tissues were then harvested, frozen in liquid nitrogen, and stored at -80 °C until further use. The iron solubilizing capacity of root washings collected was determined spectrophotometrically through a phenanthroline based colour reaction as described by Takagi (1976). Absorbance was recorded at 508 nm and the concentration of solubilized iron was determined according to Beer-Lambert's law.

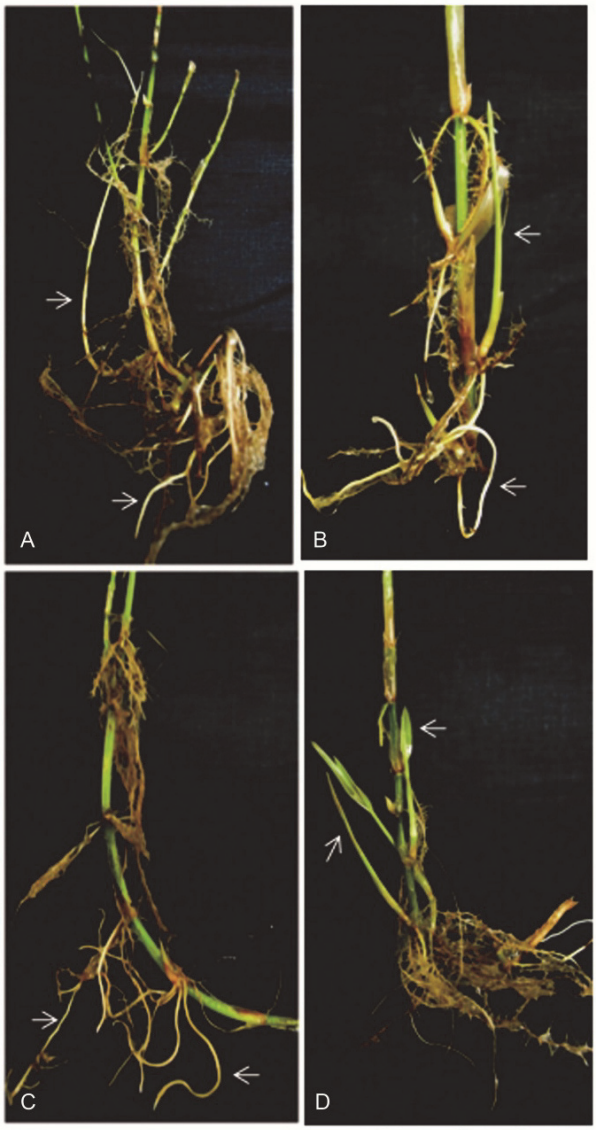

Fig. 1. The phenotype of *Porteresia coarctata* plants grown in a half-strength MS medium. *A* - pH 7.9, 150 mM NaCl; *B* - pH 7.9, 0 mM NaCl; *C* - pH 5.7, 150 mM NaCl; *D* - pH 5.7, 0 mM NaCl;. The *arrows* indicate new roots and leaves emerging from a rhizome.

Young leaves were harvested and the genomic DNA was extracted by using a modified cetyl trimethyl-ammonium bromide (CTAB) method (Michiels *et al.* 2003). A PCR reaction was carried out using the *P. coarctata* genomic DNA as template and degenerate primers *IDEF1 ORF Fwd1* and *IDEF1 ORF Rev1*, which were designed in the conserved B3 DNA binding domain region of rice *IDEF1*. The PCR product obtained showed a maximum identity to *IDEF1* (BR000654.1) in *BLAST* analysis. Subsequently, the full-length gene and cDNA of the clone were isolated by the thermal asymmetric interlaced PCR method (Liu *et al*. 2005) and sequenced. The detailed description of the methodology is given in the Supplement. Primers used in the study are listed in Table 1 Suppl. An open reading frame (ORF) was identified using the *ORF* finder at *NCBI* (http://www.ncbi.nlm.nih.gov/). The longest ORF with predicted start and stop codons was identified as a putative coding region. Sequence alignment was done using the *ClustalW* program at the European Bioinformatics Institute and phylogenetic analysis was carried out using the neighbor-joining method in the *Mega 6* program (Tamura *et al*. 2013). A conserved domain present in the ORF was identified using the conserved domain database (Marchler-Bauer *et al*. 2009).

For Southern hybridization, the genomic DNA from *P. coarctata* was isolated as described by Michiels *et al*. (2003). The DNA was digested with *BclI*, *EcoRI*, or *HindIII,* separated on a 1.0 % (m/v) agarose gel (25 µg each), and alkali-transferred onto a nylon memebrane (*Hybond-*N^+, *GE Healthcare Life Sciences*, USA). The membrane was hybridized with dCTP [α-^{32}P]-labeled *Sau3AI* fragment of *PcIDEF* containing a conserved B3 DNA binding domain and a 250 bp 3' UTR fragment according to Sambrook *et al*. (1989). The membrane was visualized using autoradiography.

For *in vivo* localization of *PcIDEF1*, the *PcIDEF1* ORF without the stop codon containing *KpnI* and *HindIII* restriction sites at its 5' and 3' ends, respectively, was amplified by PCR using *PcIDEF1-KpnI* forward and *PcIDEF1-HindIII* reverse primers (Table 1 Suppl.). The PCR product was then subcloned into the *pBS-SK-green fluorescent protein* (*GFP*) vector at *KpnI* and *HindIII* sites to yield the construct *pBS-SK-PcIDEF1-GFP*. The *PcIDEF1-GFP* fusion product was then excised from the *pBS-SK-PcIDEF1-GFP* vector using *KpnI* and *SacI* enzymes and inserted at the same sites in the *pCAMBIA 1301* vector under the double strength 35S CaMV promoter. The resulting construct *pPcIDEF1-GFP-1301* was introduced in tobacco (*Nicotiana tabacum* L.) cv. Petit Havana by *Agrobacterium* mediated transformation (Horsch *et al.* 1985). A transgenic tobacco plant harbouring the *35S-GFP-1301* construct was used as control. Epidermal leaf peels and roots mounted in water were prepared from the transgenic tobacco lines and examined under a confocal laser scanning

microscope (CLSM, *Leica TCS-SP2-RS*, Wetzlar, Germany).

Electrophoretic mobility shift assay (EMSA) was performed using hexa-histidine (His-6)-tagged PcIDEF1 as candidate protein and 67 bp IDE1 duplex (Kobayashi *et al*. 2007) as probe. Briefly, the biotin-labeled IDE1 probe was incubated with 2 μg of the (His-6)-tagged PcIDEF1 protein (see Suppl.) in a reaction mixture containing 10 mM Tris (pH 7.5 at 20 °C), 50 mM KCl, 1 mM dithiothreitol, 2.5 % (v/v) glycerol, 5 mM $MgCl_2$, 0.05 % (m/v) *Nonidet P-40* and 50 μg cm^{-3} of polydeoxy-inosinic-deoxycytidylic acid (dI-dC) as non-specific competitor at room temperature for 30 min. In the competition experiment, a 200-fold molar excess of ice cold unlabelled *IDE1* as specific competitor was added to the mixture before the start of the binding reaction. The free and protein-bound probes were separated on a 5 % (m/v) polyacrylamide gel and electrophoretically transferred to a nylon membrane. The shifted bands were visualized using a chemiluminescent nucleic acid detection kit (*Pierce*, Rockford, USA) according to the manufacturer's instructions.

For binding to DNA elements present in the *PcIRT1* 5' upstream region, the PcIDEF1 protein (2 μg) was incubated with dATP [α-^{32}P]-end-labeled DNA fragments taken from the *PcIRT1* 5' upstream region (Table 2 Suppl.) in the reaction mixture as described above. For a competition experiment, a 200-fold excess of unlabeled fragments and 50 μg cm^{-3} of poly dI-dC were used as specific and non-specific competitors, respectively. The His-6-tagged thioredoxin protein was used as negative control to confirm that binding the protein to the probe was only due to PcIDEF1. The free and protein bound probes were separated on a 10 % polyacrylamide gel which was later dried and exposed to an X-ray film.

For immobilized metal ion affinity chromatography (IMAC) assay, the nitrilotriacetic acid (NTA)-agarose (*Qiagen*, Valencia, USA) was charged with Ni^{2+} by using a 2 % (m/v) $NiSO_4$ solution according to the manufacturer's instructions. For charging with Fe^{2+}, the NTA resin was incubated with a mixture of 2 % (m/v) $FeSO_4$ and 150 mM sodium ascorbate solution in the dark at 4 ºC in order to prevent a rapid oxidation of Fe^{2+} to Fe^{3+} (Kobayashi *et al*. 2012). After charging, 0.25 cm^3 of the NTA resin was packed in a column, and the storing buffer was allowed to drain out. The column was subsequently washed with 10 bed volumes of deionized water to wash off the unbound metal ions. The column was then re-equilibrated with 10 bed volumes of a 1× lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, and 10 mM imidazole). After the wash, 1 cm^3 of isopropyl β-D-1-thiogalactopyranoside (IPTG) induced protein solution in the 1× lysis buffer was loaded to the column and the flow-through was collected. The unbound and bound proteins were later serially washed out and collected as fractions by applying 1 cm^3 of the 1× lysis buffer containing 20, 50, 250, and 1000 mM imidazole. For Fe^{2+} binding assay, the 1× lysis buffer was supplemented with 25 mM sodium ascorbate in order to prevent oxidation of Fe^{2+} to Fe^{3+} (Kobayashi *et al*. 2012). An aliquot of each fraction (0.04 cm^3) was subjected to SDS-PAGE and visualized by Coomassie brilliant blue (R250) staining.

The total RNA from treated and control *P. coarctata* plants was extracted using a *TRI* reagent (*Sigma-Aldrich*, St. Louis, USA) according to the manufacturer's instructions. The first strand cDNAs were synthesized from the total RNA using *BioScript* reverse transcriptase (*Bioline*, town?, USA). Quantitative real time PCR was performed using *SYBR Premix Ex Taq*™ *II* (*TaKaRa*, Tokyo, Japan) in a *Step One Plus*™ real time PCR system according to the manufacturer's instructions (*Applied Biosystems*, Taunton, USA). The PCR cycling condition was as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a 96-well optical reaction plate (*Applied Biosystems*). A housekeeping gene *Pcβ-Actin* was used as endogenous control. Each reaction was performed in triplicate. The experiment was repeated thrice and statistically analyzed in order to evaluate data reproducibility. The relative gene expression was calculated by the comparative C_T (ΔΔCt) quantitation method using the formula $2^{-\Delta\Delta Ct}$. A partial cDNA of an iron regulated transporter sharing a high identity to *OsIRT1*, designated as *PcIRT1*, was used as marker transcript to verify the iron deficiency response in *P. coarctata.*

The data represent means ± SEs of three independent experiments. Statistical significance was tested using one way *ANOVA* with the Tukey HSD (honest significant difference) method.

Results

The young plants of *P. coarctata* developed new roots after one month in the hydroponic culture. The absence of iron in the hydroponic medium at pH 5.7 did not result in any visual iron deficiency symptom, such as leaf chlorosis, up to three weeks after which the leaves started turning pale yellow. The plants were able to grow well (without showing symptoms of salt injury such as leaf curling or yellowing) up to 200 mM NaCl for 21 d. Also, the addition of NaCl (100, 150, and 200 mM) to the iron deficient medium did not result in any of the symptoms related to iron deficiency or salt toxicity (Fig. 1 Suppl.). Plant growth was also compared at pH 5.7 and pH 7.9. Both the presence of 150 mM NaCl in the medium and alkaline pH facilitated growth of young roots in *P. coarctata* compared to the control conditions (pH 5.7 without 150 mM NaCl) though the emergence of new leaves from rhizomes was more evident at pH 5.7 in the absence of NaCl (Fig. 1). The iron deficiency and NaCl

treatment when given either separately or in combination did not significantly change iron accumulation in root tissues (Fig. *2A*). However, remarkably higher iron content in the unwashed roots grown in the half strength MS compared to the washed roots suggested for a proper removal of apoplastic iron during the sample harvesting process (Fig. 2*A*). Iron accumulation in leaves was comparatively lower in all the treatments including the control or iron sufficient medium. Chlorophyll content was not affected by the treatments as compared to the control (Fig. 2*B*). The results from the hydroponic assay for the NaCl tolerance limit and iron deficiency response therefore suggest that *P. coarctata* was able to grow up to 200 mM NaCl with an optimum growth at 150 mM NaCl based on the visual observation of the plants (Fig. 1 Suppl.).

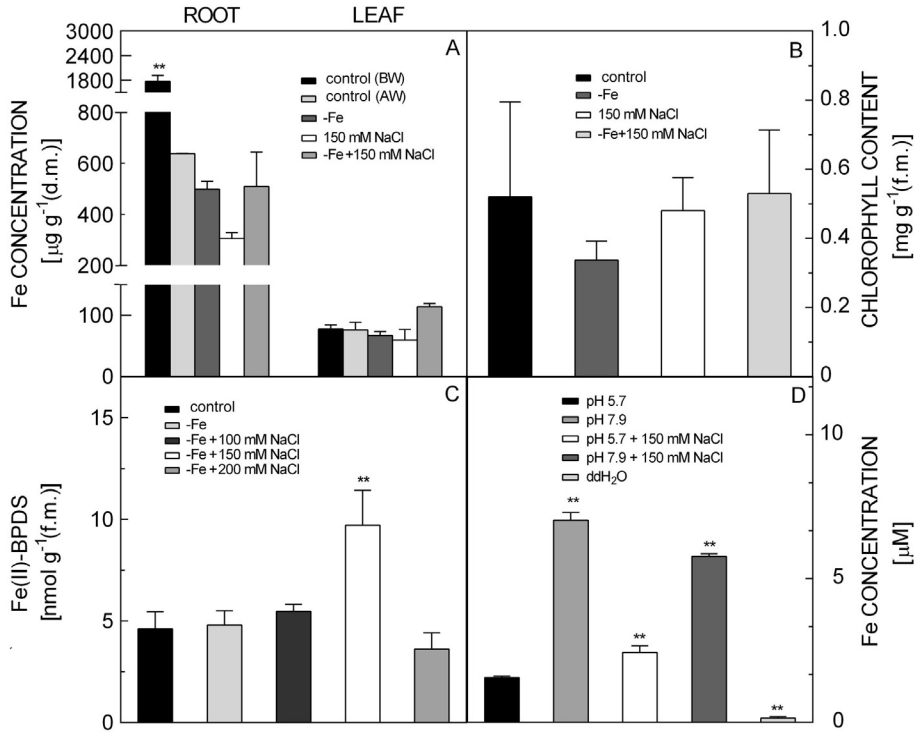

Fig. 2. The responses of *Porteresia coarctata* to iron deficiency, NaCl, and alkaline pH. *A* - Fe accumulation in roots and leaves from MS medium determined before (BW) and after (AW) washing the roots Fe deficient, 150 mM NaCl and Fe deficient with 150 mM NaCl containing medium. *B* - Chlorophyll content in leaves of plants grown in control, Fe deficient, 150 mM NaCl, and Fe deficient with 150 mM NaCl MS media. *C* - Ferric chelate reductase assay in plants treated with Fe deficiency, Fe deficiency plus 100, 150, and 200 mM NaCl. *D* - Iron solubilization assay in washings from roots grown at pH 5.7 or 7.9, with or without 150 mM NaCl and double distilled water as control. Means ± SEs of three independent experiments. Significant differences compared to the control conditions were calculated using one way *ANOVA* with Tukey's post-hoc HSD test (** - $P < 0.01$).

Rice, though a grass species, possesses a fully functional Fe^{2+} transport system but a low activity of endogenous Fe^{3+} reductase, which is possibly an adaptation to the flooded rice field (Ishimaru *et al*. 2006). Since the natural habitat of wild rice *P. coarctata* is inundated twice a day with sea water and remains in submerged conditions for almost 12 hours (Jagtap *et al*. 2006), we wanted to check the existence of the strategy I machinery in it if not fully, at least partially by assessing the activity of FRO. To our surprise, *P. coarctata* showed a high Fe^{3+} reductase activity in response to iron deficiency combined with the salt stress at pH 5.7. The root FRO assay revealed a significant increase in activity in the iron deficient with 150 mM NaCl treated plants as compared to the control plants (Fig. 2*C*). At the same time, the iron solubilization capacity of the *P. coarctata* root washings (the strategy II mechanism) was also high for the pH 7.9 and pH 7.9 with 150 mM NaCl treated plants (Fig. 2*D*). The above results exemplify that *P. coarctata* might possess an active strategy I machinery besides the existence of a fully functional strategy II mechanism.

Cloning and sequencing amplified product obtained using the *P. coarctata* genomic DNA resulted in a partial sequence of about 1.5 kb showing maximum identity to the rice *IDEF1* transcription factor. To obtain the remaining sequence information, two rounds of thermal asymmetric interlaced PCR were performed, which generated a full length product of 4.265 kb. The product was designated as *PcIDEF1* (JN615010.1). The *PcIDEF1* gene was 3.505 kb long with nine exons and eight introns (Fig. 2 Suppl.).

Reverse transcriptase PCR for isolation of the *IDEF1* cDNA from *P. coarctata* using the gene sequence

resulted in a product of 1 460 bp designated as *PcIDEF1* cDNA (GenBank acc. No. JN615009.1, predicted protein product sequence acc. No AEX88464.1). Database searches reveal that the PcIDEF1 polypeptide shared maximum identities to B3 DNA binding domain containing proteins, which belong to the plant-specific transcription factor family ABI3/VP1 (Kobayashi *et al.* 2007). *In silico* analysis also revealed a metal binding

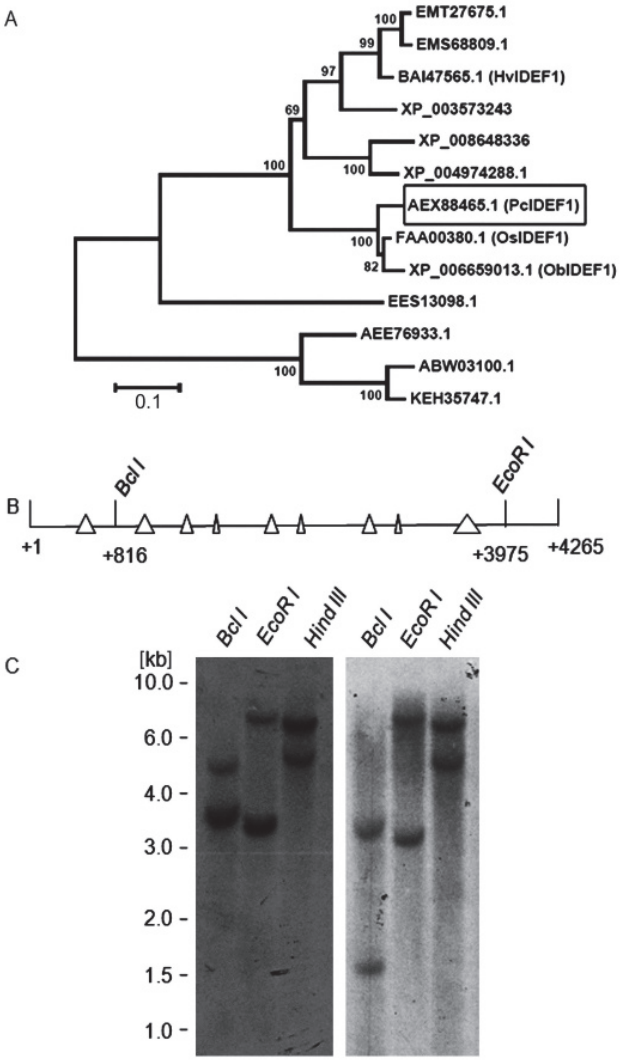

Fig. 3. *In silico* characterization of PcIDEF1. *A* - A neighbour joining tree of PcIDEF1 with other B3 binding domain containing proteins from graminaceous and non-graminaceous species with 10 000 bootstrap replicates. AEX88465.1 (*Porteresia coarctata*), XP_008648336 (*Zea mays*), XP_003573243 (*Brachypodium distachyon*), FAA00380.1 (*Oryza sativa*), BAI47565.1 (*Hordeum vulgare*), EES13098.1 (*Sorghum bicolor*), EMS68809.1 (*Triticum uratu*), AEE76933.1 (*Arabidopsis thaliana*), XP_006659013.1 (*Oryza brachyantha*), EMT27675.1 (*Aegilops tauschii*), XP_004974288.2 (*Setaria italica*), ABW03100.1 (*Pisum sativum*), KEH35747.1 (*Medicago truncalata*). The analysis shows PcIDEF1 clustering with IDEF1 from *Oryza sativa* and *Oryza brachyantha*. *B* - A restriction map of the *PcIDEF1* gene. The *triangles* represent exons and the *lines connecting the triangles* represent introns. *C* - Copy number analysis of the *PcIDEF1* gene in *P. coarctata* genome by Southern blot hybridization; hybridization with α ^{32}P-labeled *Sau3AI* fragment of *PcIDEF1* ORF (*on the left*) and hybridization with α ^{32}P-labeled *PcIDEF1* 3' UTR fragment (*on the right*).

domain with histidine-asparagine (HN) repeat towards the N terminal end of PcIDEF1 (Fig. 2 Suppl.). PcIDEF1 is closely related to *Oryza sativa* OsIDEF1 sharing an 85 % amino acid identity at the whole protein level and as high as 98 % identity with respect to the B3 domain (Fig. 2 and 3*A* Suppl.).

The copy number of *PcIDEF1* in *P. coarctata* was assessed by Southern hybridization. Three enzymes, namely *BclI*, *EcoRI*, and *HindIII*, were chosen to digest the *P. coarctata* genomic DNA. The restriction enzymes chosen did not have any sites in the *PcIDEF1* cDNA sequence. However, in the genomic sequence, the *BclI* enzyme cut the *PcIDEF1* gene at 816 nt position within the first intron, whereas *EcoRI* cut it at 3 975 nt position

in the 3' flanking region after the last exon (Fig. 3*B*). There were no restriction sites for *HindIII* in the entire *PcIDEF1* genomic fragment. Two bands were observed in *BclI*, *EcoRI*, and *HindIII* digested DNAs when probed with a *Sau3AI* digested *PcIDEF1* ORF fragment (Fig. 3*C*). Reprobing the blot with the 3' UTR fragment also produced two bands in each lane (Fig. 3*C*). The bands visible in the *BclI* digested lane, when probed with the *PcIDEF1* ORF fragment, were approximately of 5 and 3.5 kb sizes (Fig. 3*C*), which was in accordance with a 3 505 kb size of the *PcIDEF1* gene. However, the presence of an approximately 1.5 kb band, which is much below the size of the *PcIDEF1* gene (Fig. 3*C*) in the *3' UTR* probed *BclI* digested lane, depicts a possibility for existence of one more *BclI* site either in the 4th intron or the 5th exon (~ 2 026 bp) in the second paralog of the *PcIDEF1* gene. In *EcoRI* and *HindIII* digested lanes, band sizes were either close to 3.5 kb or above that,

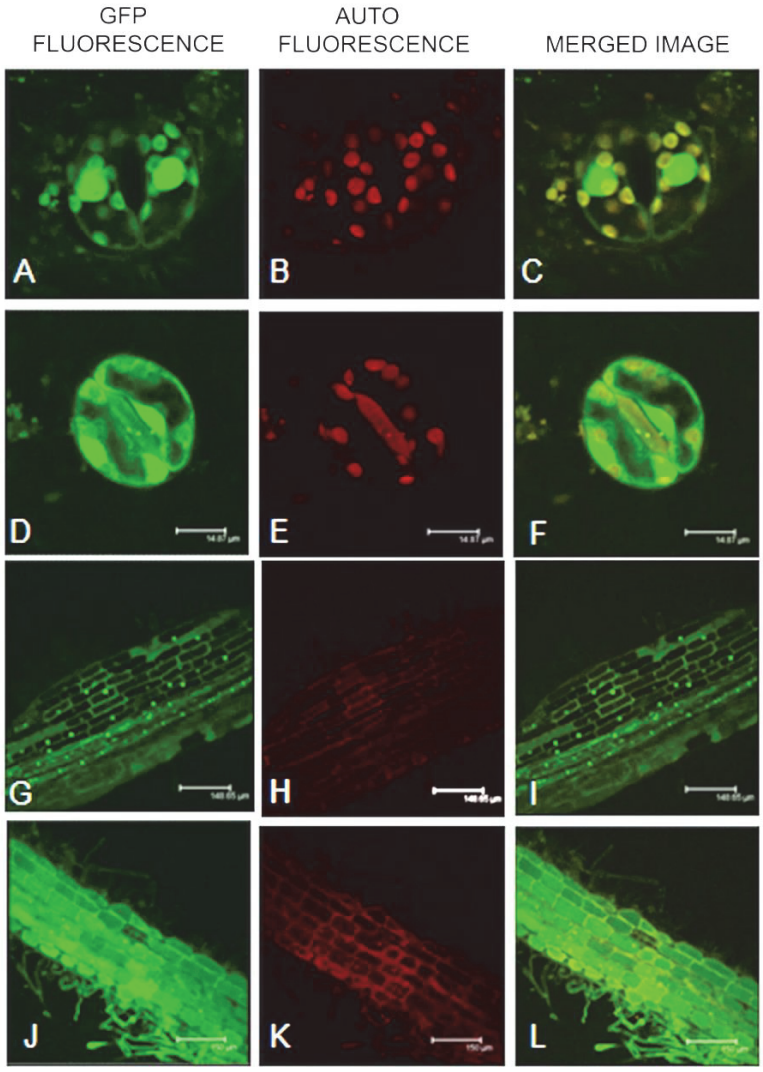

Fig. 4. Subcellular localization of PcIDEF1. Confocal laser scanning microscopy imaging of epidermal leaf peels prepared from transgenic tobacco expressing PcIDEF1-green fluorescent protein (GFP) fusion protein as test *(A,B,C)* and GFP as control (*D,E,F*). Longitudinal sections of root tissues prepared from transgenic tobacco expressing a PcIDEF1-GFP fusion protein as test (*G,H,I*) and GFP as control (*J,K,L*).

which was either the same or more with respect to a 3.505 kb size of the *PcIDEF1* gene. From the above results, it was concluded that the *PcIDEF1* gene has a close paralog in the *P. coarctata* genome.

The *in vivo* localization studies using transgenic tobacco expressing the PcIDEF1-GFP fusion protein revealed that the expression of PcIDEF1 was restricted to the nucleus as evident from nucleus-specific fluorescence both in roots and in guard cells (Fig. 4*A*,*B*). In contrast, fluorescence corresponding to GFP was localized both in the cytoplasm and in the nucleus of root and guard cells.

The full-length PcIDEF1 protein containing the B3 DNA binding domain towards the C-terminus was heterologously expressed in *E. coli* and purified as fusion

to His-6-tagged thioredoxine (Fig. 5*A*). Electrophoretic mobility shift assay was performed using the purified protein and a 67 bp synthetic oligonucleotide *IDE1* (Kobayashi *et al*. 2007) to check for their binding specificity. The result shows a broad shifted band, which corresponded to 2 μg of the PcIDEF1 fusion protein with 20 fmol of the biotin labeled *IDE1* element (Fig. 5*B*). Competition with a 200-fold molar excess of unlabelled *IDE1* (a specific competitor) completely eliminated the shifted DNA-protein complex (Fig. 5*B*), whereas the presence of the non-specific competitor poly (dI-dC) in all the samples did not alter binding of PcIDEF1 to *IDE1* element indicating the specificity of binding (Fig. 5*B*).

Additionally, the putative 5' upstream region (1 744 kb) of the *P. coarctata iron regulated transporter 1* (*PcIRT1)* gene encompasses six *IDE* like elements. Based on the distribution of *IDE* like elements, the 5' upstream region was divided into five fragments (1, 2, 3, 4, and 5), out of which fragment 1 contained both the *IDE1* element and the *IDE2* element, fragments 4 and 5 consisted of the *IDE1* like elements, and fragments 2 and 3 consisted of the *IDE2* elements (Table 2 Suppl.). Electrophoretic mobility shift assay was performed to check the binding ability of PcIDEF1 to these fragments. This assay with individual *IDE* elements shows clear binding specificities to the *IDE1* element containing fragments (fragments 1, 4, and 5) and no binding to those with *IDE2* (Fig. 5*C*). The same probe did not show any shifted band when incubated with negative control protein thioredoxine (Fig. 5*C*). Competition with a 200-fold molar excess of unlabelled specific fragments reduced the signal intensity of the shifted bands considerably (Fig. 5*C*). In the case of fragment 4, specific binding by PcIDEF1 was revealed by a reduced signal intensity of the unbound radio-labelled probe at the bottom (Fig. 5*C*), whereas the signal intensity for the same unbound radio-labeled probe remained high in the presence of specific competitor (200 fold molar excess of unlabelled fragment 4), thioredoxine protein, and in the absence of PcIDEF1 (Fig. 5*C*). There was no change in band intensity with the presence of a non-specific competitor poly (dI-dC) in all the samples.

To check the binding efficiency of the HN domain of the PcIDEF1 protein to divalent cations, immobilized metal affinity chromatography (IMAC) assay was performed. Since histidine binds to divalent cations (Ueda *et al*. 2003), we included a His6-tagged thioredoxine protein in the assay as positive control protein and glutathione-S-transferase (GST) was used as negative control. The result shows the divalent cations like Fe^{2+} and Ni^{2+} were bound to the PcIDEF1-GST fusion protein and to His-6-tagged thioredoxine (the positive control), whereas GST (the negative control) itself did not bind to any of them (Fig. 6*A*,*B*). The

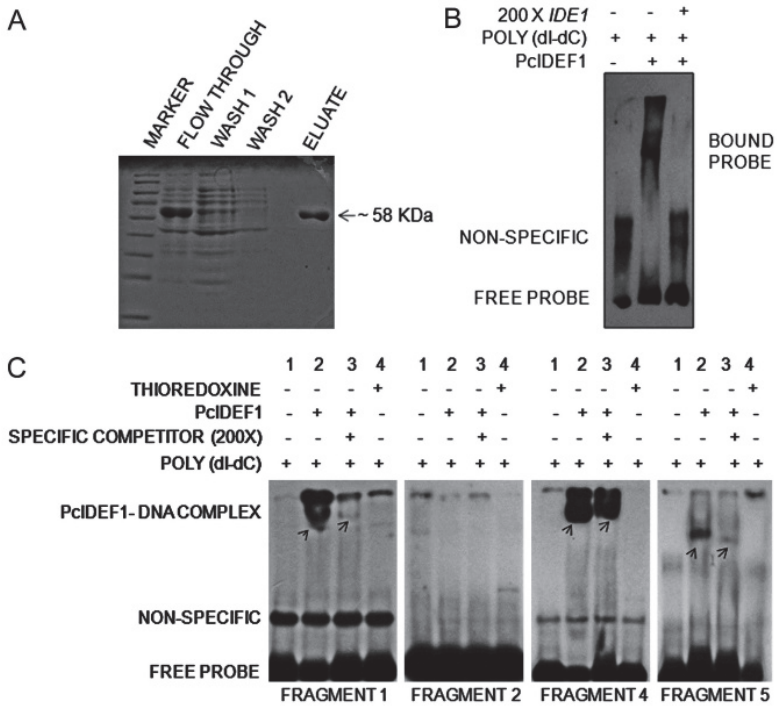

Fig. 5. Electrophoretic mobility shift assay demonstrating binding of PcIDEF1 to *IDE1* like elements *in vitro*. *A* - Purification of the His-6-tagged PcIDEF1 protein expressed in *Escherichia coli. B* - PcIDEF1 binding to 67 bp *IDE1* element. *Lane 1* contains 20 fmol of biotin-*IDE1* probe without protein. *Lane 2* contains a biotin-*IDE1* probe incubated with 2 μg of the His-6-tagged PcIDEF1 protein. The competition experiment was carried out by adding 200 fold molar excess of unlabeled *IDE1* (*lane 3*). *C* - Binding of PcIDEF1 to 5' upstream region fragments (1, 2, 4, and 5) of the *PcIRT1* gene containing *IDE* like elements. Fragments 1, 4, and 5 contain the *IDE1* element and fragment 2 contains the *IDE2* element. The *arrows* indicate shifted bands. *Numbers* represent lanes.

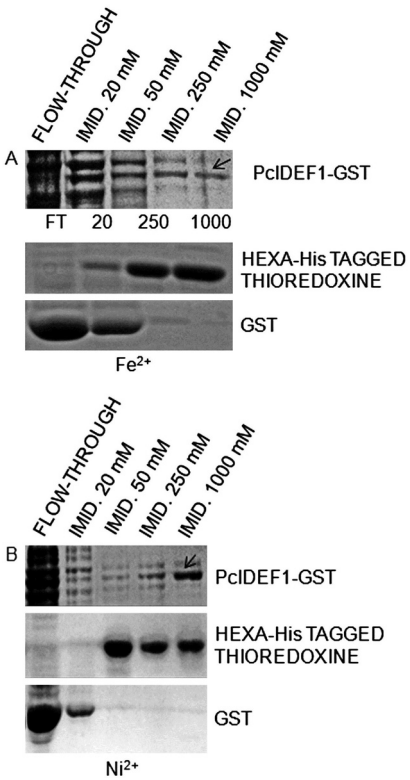

Fig. 6. Metal binding assay using immobilized metal ion affinity chromatography. The PcIDEF1-glutathione-S-transferase (GST) fusion protein, a GST protein as negative control, or a His-6-tagged thioredoxine protein as positive control were loaded to $FeSO_4$ (Fe^{2+}) (*A*) or $NiSO_4$ (Ni^{2+}) (*B*) charged nitrilotriacetic acid agarose resins. The columns were washed to separate unbound protein fractions. Bound proteins were then eluted with an increasing concentration of imidazole (IMID). Aliquots of the purified fractions were analyzed by SDS-PAGE and visualized by Coomassie brilliat blue staining.

PcIDEF1-GST fusion protein was detected even at higher concentrations of imidazole (1 000 mM), whereas free GST was detected in the flow through. The result shows Ni^{2+} was more efficiently bound as compared to Fe^{2+}.

Transcription of *PcIDEF1* was higher in roots of the iron deficiency treated plants compared to the control. The addition of NaCl to the iron deficient medium further increased the *PcIDEF1* transcription. However, the increase in transcription was similar to the iron deficiency treatment alone when 200 mM NaCl was added. All the changes in expression were significantly different from the control conditions in both root tissue and leaf tissue with the exception in roots of the 200 mM NaCl treated plants and in leaves of the 100 mM NaCl treated plants (Fig. 7*A*). Transcription of *PcIRT1*, a homolog of iron deficiency inducible *AtIRT1* and *OsIRT1* (Eide *et al*. 1996, Bughio *et al*. 2002) was also induced in response to the iron deficiency and 150 mM NaCl, both in combination and in isolation (Fig. 7*B*), further validating upregulation of *PcIDEF1* under the iron deficiency and salinity.

We also tested the molecular response of *P. coarctata* to alkalinity by comparing the mRNA expression of *PcIDEF1* and *PcIRT1* genes at pH 5.7 and 7.9 both in the presence and in the absence of NaCl. The result shows a clear tendency for *PcIDEF1* upregulation induced by alkalinity although not significant across all the treatments (Fig. 7*C,D*). Lack of a significant change in gene expression could be attributed to a possible post transcriptional regulation, which comes into play under iron deficiency conditions and other environmental stresses. Interestingly, *PcIRT1* expression in response to the above mentioned treatments exactly paralleled the mRNA accumulation profile of *PcIDEF1* in root tissue (Fig. 7*C*), whereas in leaf tissue, it followed a completely reverse pattern to that of *PcIDEF1* (Fig. 7*D*).

Discussion

Porteresia coarctata being a close relative of rice and an inhabitant of coastal wetlands is a potential model for studying stress biology (Sengupta and Majumder 2010). *Porteresia* grows in alkaline soil (Jagtap *et al*. 2006) and the harmful effects of soil alkalinity are related to the non-availability of nutrients, particularly iron (Lindsay *et al*. 1984). The critical concentration of soluble iron for plants is approximately 10^{-8} M, whereas in a pH range of 7.4 to 8.5 (characteristic of *Porteresia* beds), iron solubility drops to 10^{-10} M (Lindsay *et al*. 1984). Also, high concentrations of NaCl disrupt potassium, iron, and other mineral nutrients and create osmotic stress, which results in secondary problems such as oxidative stress (Zhu 2001). *P. coarctata* growing in such habitats may thus be subjected simultaneously to high salinity and iron deficiency.

In order to assess the effect of NaCl and iron deficiency on morphology and growth of *P. coarctata* plants, we performed hydroponic assays using young plants emerging from rhizomes as it is difficult to establish seedlings in this species (Flowers *et al*. 1990, Sengupta and Majumder 2010). In our observation, the young plants were able to tolerate the iron deficiency and NaCl up to the 200 mM concentration when the treatment was given alone or in combination, without showing any leaf chlorosis and growth abnormalities. New roots and leaves originated from rhizomes in the plants subjected to these treatments (Fig. 1, Fig. 1 Suppl.). The absence of morphological symptoms related to the combined effect of NaCl and iron deficiency in this study is contrary to earlier observations in *Medicago ciliaris* (Rabhi *et al*. 2007) and barley (Yousfi *et al*. 2007). It is possible that NaCl is secreted through hairs present in the leaf lamina (Flowers *et al*. 1990) or transferred into vacuoles due to accumulation of compatible solutes in cytoplasm (Sengupta and Majumder 2010) and hence, the young plants of *P. coarctata* did not show any growth defects. The absence of leaf chlorosis may be attributed to the

presence of local iron that is embedded in root apoplast; it can be remobilized for plant growth under iron deficient conditions for a short period (Gregory *et al*. 2003). However, the fact that *P. coarctata* was able to withstand the iron deficiency and NaCl treatments for a period of three weeks indicates the existence of further mechanisms. In this regard, the importance of a highly differentiated underground rhizome system (Sengupta and Majumder 2010) for the time and duration of stress tolerance should also be considered. Also, it is interesting to note that *P. coarctata* possesses fully functional FRO (a component of strategy I mechanism), which showed a high activity in the absence of iron together with 150 mM NaCl in the medium (Fig. *2C*), besides exhibiting a grass species specific high iron solubilizing ability of the root exudates at pH 7.9 (Fig. *2D*), underlining the possibility of both strategy I mechanism and strategy II mechanism operating in tandem, at least under wetland conditions.

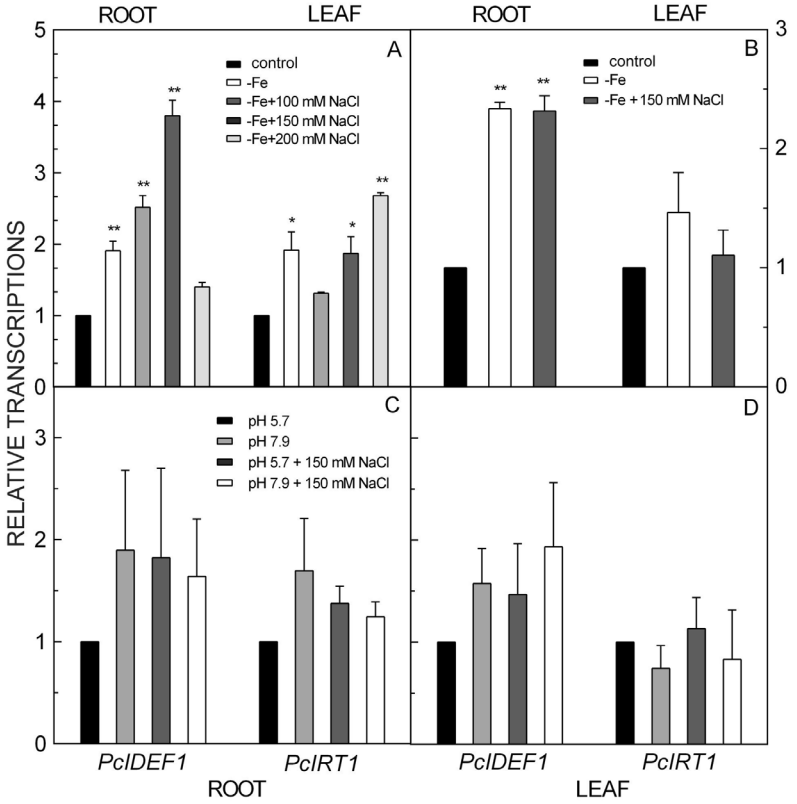

Fig. 7. Effects of iron deficiency and NaCl on *PcIDEF1* and *PcIRT1* transcript abundance in *Porteresia coarctata*. *A* - The relative abundance of *PcIDEF1* transcripts in root and leaf tissues of *P. coarctata* grown in iron deficiency (-Fe), or in iron deficiency with incremental NaCl concentrations (-Fe + 100 mM NaCl; -Fe + 150 mM NaCl; -Fe + 200 mM NaCl) for 21 d. *B* - The relative abundance of *PcIRT1* transcripts in root and leaf tissues of *P. coarctata* treated with iron deficiency (-Fe), or in iron deficiency with 150 mM NaCl (-Fe + 150 mM NaCl) for 21 d. *C,D* - The relative abundance of *PcIDEF1* and *PcIRT1* transcripts in *P. coarctata* plants grown in a medium with pH 5.7 or pH 7.9, either with or without 150 mM NaCl for 21 d in root and leaf tissues. The control is set to 1. Means ± SEs of three independent experiments. Significant differences compared to the control conditions are marked with * ($P < 0.05$) and ** ($P < 0.01$).

Since *P. coarctata* is wetland plant where soil is alkaline and saline, we wanted to understand the mechanisms of its tolerance to iron deficiency conditions that may arise. We focused on the IDEF1 transcription factor as it has been well studied in rice to which *P. coarctata* is closely related (Lu and Ge 2004) and is a central iron deficiency regulator that can confer iron deficiency tolerance in plants (Kobayashi *et al*. 2007, 2009, 2013). The PcIDEF1 protein was 16 amino acids shorter than its rice homolog OsIDEF1 and possessed a shorter histidine-asparagine stretch at its 5' end (Fig. 2 Suppl.). A similar observation has been reported for genes in salt cress (*Thellungiella halophila*), a halophyte, and its close relative *Arabidopsis* (Taji *et al*. 2004). The nuclear localization of the PcIDEF1-GFP fusion protein (Fig. 4) and its binding to the *IDE1* like element due to the presence of the B3 domain at the C-terminal end (Fig. 5*B*) suggest its function as transcription factor.

PcIDEF1 bound Fe^{2+} moderately and Ni^{2+} strongly as 1 M imidazole was able to displace the protein, albeit it was detectable in a small amount in 20 mM imidazole fractions, too. Moderate binding PcIDEF1 to Fe^{2+} could be due to the shorter HN stretch. In *P. coarctata* the HN region has six histidine residues, whereas in OsIDEF1 it is a longer stretch. However in both OsIDEF1 and PcIDEF1, the HN region is flanked by proline regions

unlike in HvIDEF1 where the HN region is shorter than both OsIDEF1 protein and PcIDEF1 protein (Fig. 2 Suppl. and Kobayashi *et al*. 2012). It may also be noted that barley is iron deficiency tolerant in spite of inefficient binding to iron attributable to a very short HN region in HvIDEF1 because it secretes different types of mugineic acids in large amounts in response to iron limiting conditions (Takahashi *et al*. 2001, Suzuki *et al*. 2008). Study on iron solubilization capacity in the root zone of *P. coarctata* at acidic and alkaline pH shows a higher concentration of solubilized iron at alkaline pH (Fig. 2*D*). It is possible that *P. coarctata* might involve root exudates to solubilize iron as has been reported in oat (Takagi 1976). In addition to that, direct binding PcIDEF1 to iron and a higher transcription of *PcIDEF1* at pH 7.9 (Fig. 7*C*,*D*) in the presence or absence of NaCl clearly suggest the existence of subtle iron sensing and uptake mechanisms in *P. coarctata* growing in alkaline and saline soils. However, it is surprising that even at pH 5.7 and in the absence of iron *P. coarctata* did not show chlorotic symptoms for as long as three weeks, suggesting a sufficient tissue iron reserves. Iron storage and maintenance of homeostasis inside the plant is critical in terms of releasing iron during times of its sub-optimal supply (Jeong and Guerinot 2009).

PcIDEF1 expression studies demonstrate that it was responsive to iron deficiency and NaCl in the growth medium. Interestingly, in the experiment where the combined treatment of the iron deficiency and 200 mM NaCl was given, there was a marked increase in *PcIDEF1* transcription in leaves but significant decrease in roots (Fig. 7*A*). Since *PcIDEF1* expression remained high in both roots and leaves up to 150 mM NaCl added to the iron deficient medium, in addition to a healthy morphology (Fig. 1 Suppl.), this concentration of NaCl was considered to be optimal for plant growth with respect to iron deficient conditions. The salinity or iron deficiency alone or in combination increased *PcIDEF1* transcription suggesting that it was responsive to both iron deficiency and NaCl. Moreover, up-regulation of *PcIRT1*, a *P. coarctata* homolog of iron deficiency inducible *Arabidopsis IRT1* (Eide *et al*. 1996) by more than two fold similar to that of *PcIDEF1* validates the iron deficiency treatment conditions imposed, besides indicating the existence of both strategy I and II mechanisms. The results obtained in the present study are different from those observed in rice where *OsIDEF1* is constitutively expressed in leaf and root tissues in both iron deficient conditions and iron sufficient conditions (Kobayashi *et al*. 2007). It is possible that it is a consequence of a shorter stretch of HN repeats near the N terminus with intact N and C terminal proline regions as compared to OsIDEF1. Kobayashi *et al*. (2012) demonstrated that IDEF1 homologs from graminaceous species that possess HN regions of different lengths show varying degrees of binding specificities for iron. Such differences could result in a subtle regulation of tolerance effectors or pathways (Zhu 2001), rendering *P. coarctata* to respond differently from rice to iron deficiency conditions that may prevail in alkaline and saline soils. The difference may also be attributed to contrasting habitats, difference in ploidy, and ecosystem dynamics of both the species. Induction of *PcIDEF1* transcription in iron deficient conditions alone or together with NaCl suggest the existence of a key role for *PcIDEF1* in controlling regulation of iron-responsive genes, which may be highly specific to iron deficiency and salinity induced iron limiting conditions.

Electrophoretic mobility shift assay demonstrated binding of PcIDEF1 to the *IDE1* like elements present in the 5' flanking region of the *PcIRT1* gene, showing strongest binding to fragment 4 (Fig. *5C*). It may be noted that the core sequence of fragment 4 is CATGCATG where two repeats of the *IDE1* elements were present overlapped at the centre (GC) from both the ends forming a palindromic sequence. The fact that the promoter regions of many iron-deficiency-inducible genes in barley, rice, *Arabidopsis*, and tobacco possess *IDE* like sequences suggest the existence of a partially conserved IDE-mediated gene regulation mechanism for iron uptake among graminaceous (strategy II) and non-graminaceous (strategy I) species (Bauer *et al*. 2004, Ducos *et al*. 2005, Kobayashi *et al*. 2005). Furthermore, *PcIRT1* expression followed a similar pattern as that of *PcIDEF1* in response to the iron deficiency, alkaline pH, and NaCl around roots (Fig. 7). These findings put forward a possibility that PcIDEF1 might have a regulatory role in expression of the *PcIRT1* gene, besides suggesting a possible link between the two strategies.

In conclusion, the PcIDEF1 transcription factor isolated and characterized from *P. coarctata* in the present study is different from OsIDEF1 characterized in its relative *Oryza sativa* at both sequence and functional level. This is the first report of an *IDE1* binding transcription factor which is responsive to dual stress of iron deficiency and salinity. The results obtained in this study may form a basis for efforts to manipulate iron deficiency responses in cultivated rice under conditions of salt stress. A potential dual stress response of *PcIDEF1* can be utilized in transforming glycophytic crops.

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