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Functional screening of genes from a halophyte wild rice relative *Porteresia coarctata* in *Arabidopsis* model identifies candidate genes involved in salt tolerance

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ABSTRACT

High soil salinity is one of the most important environmental stressors, and is an increasing concern under the alarmingly global climate uncertainties. Salinity affects the growth and productivity of food crops, including rice, and ultimately threatens global food security and sustainability. Conventional breeding efforts to develop salt tolerant rice varieties utilizing its primary gene pool have resulted in limited success due to the mutligenic inheritance of salt tolerance traits. On the other hand, *Porteresia coarctata*, the only halophyte wild rice, offers tremendous opportunities to improve salt tolerance in rice. However, reports on the characterization of its rich genetic resources are scanty. The present study was undertaken to functionally identify salt responsive genes of *P. coarctata* in *Arabidopsis* model using a cDNA overexpression (COX) strategy. COX-mediated hunting of transgenic *Arabidopsis* expressing a cDNA library of *P. coarctata* under control of a maize ubiquitin promoter identified 12 candidates that were involved in salt tolerance for seed germination, and seedling and reproductive growth. Of these, four genes coding for a metallothionein, a ribosomal protein, a photosystem II 10 kDa protein, and a ferredoxin:thioredoxin reductase conferred enhanced salt tolerance phenotypes, such as better root and shoot growth, and tissue tolerance by maintaining higher relative water content, membrane stability and protecting cells from reactive oxygen species. These genes could be ideal candidates for improving salt tolerance in rice; however, further studies are needed to ascertain their direct functional relevance in salt tolerance mechanisms.

1. Introduction

High soil salinity is one of the most serious environmental stressors that affects more than 77 million ha of arable land worldwide [1], with an estimated annual global cost equivalent of US$110,000 million [2]. Rice, like most other field crops, is sensitive to high soil salinity, which affects the growth and productivity of rice worldwide. Climate-related risks to fresh ecosystems will increase in the future leading to increased salinity of irrigation water due to rise in sea water levels. Salt tolerance is a quantitative trait controlled by many loci. Considerable efforts have been made to understand the complex genetic mechanisms that underlie salt tolerance through the analysis of salt stress responsive transcriptomes of the glycophyte models *Arabidopsis thaliana* and *Oryza sativa* [3].

Natural genetic variation can be exploited either through quantitative trait loci mapping [4] or altered expression of several target genes and regulators that control salt stress tolerance [5] to improve crop yield under salt stress. Salt tolerance has been reported in some traditional cultivars and land races of rice, but the development of salt tolerant varieties through breeding efforts has been slow except for a limited success reported by the International Rice Research Institute (irri.org/news/hot-topics/rice-and-climate- change). Halophytes, on the other hand, have the ability to thrive and complete their life cycle under high salinity due to their ability to exclude Na^+ at the root level, sequester Na^+ to the vacuole, exude excess salt through specialized glands in the leaves, and maintain osmotic potential through accumulation of compatible solutes [2]. Among the secondary gene pool comprising the wild relatives of *O. sativa*, *Porteresia coarctata*, a tetraploid (2n = 4x = 48) distant rice wild relative halophyte and a native of salt-marshes in S.E. Asia (coasts of Bay of Bengal), shows

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considerable adaptation to high salinity [6]. *P. coarctata*, an important coastal marsh ecosystem member in India, has an extensive rhizome system. *P. coarctata* exhibits considerably better growth, higher chlorophyll content and photosynthesis efficiency under saline conditions, and like other salt marsh plants, can withstand salinity levels as high as sea water with all known mechanisms of salt tolerance at cellular and whole plant levels [7]. It is a close wild relative of rice with KKLL genome [8] and conventional hybridization followed by embryo rescue was successfully attempted at transferring some of its useful traits to rice [9]. Genetic engineering of a few of its genes has demonstrated to confer salinity tolerance in transgenic plants [10–14], which shows it can be a potential source of genes and promoters Philipfor improvement of salt tolerance in rice.

Although both genomics and transcriptomic resources for *P. coarctata* are available [15–18], thus far, very few of its genes have been functionally characterized in rice [10,11]. Functional genomics has become an important tool in the post-sequencing era to ascertain biological function to a gene. Forward genetic screening using mutagenized populations [19] and T-DNA and/or (retro) transposon-tagged loss-of-function mutants screening [20,21] have been used for large-scale identification and characterization of genes in model plants, such as *Arabidopsis* and rice. However, high frequency of gene duplication in the genome can make loss-of-function phenotypes difficult to find. Moreover, embryonic lethality or severe developmental defects are often associated with gene knockouts [22]. To circumvent these problems, a gain-of-function strategy, i.e. activation tagging using a transcription enhancer was adopted [23], but the enhancers often resulted in complex phenotypes by affecting the expression of multiple genes. Further, T-DNA insertions are randomly distributed over the genome with preferential occurrence in gene-rich regions [24]. An alternative, yet effective approach is the efficient generation of full-length cDNAs (Fl-cDNA) and functional analysis of encoded proteins under defined experimental conditions. The cDNA library transformation approach also has the potential to produce dominant loss of function phenotypes resulting from the co-suppression of endogenous genes by overexpression of truncated or antisense cDNAs [25]. An alternative gain-of-function approach, the FOX (FL-cDNA over expresser) gene hunting system, was conceived for the expression of FL-cDNA libraries (also known as COX) [26,27]. FOX enables the comprehensive characterization of novel and important traits and the identification of causal genes where ectopic overexpression of FL-cDNA(s) generates large numbers of dominant mutations. The FOX/COX-hunting technology has been applied successfully in two plant models (i.e. *Arabidospis* and rice) because of the availability of high throughput *Agrobacterium tumefaciens*-mediated transformation systems. FOX hunting with large-scale transformation of *Arabidopsis* led to the identification of the *ESR1* gene that stimulates cytokinin independent plant regeneration [28]. Similarly, FL-cDNA libraries driven by constitutive promoters were used to generate transgenic *Arabidopsis* and rice lines that showed altered developmental traits [25–27,29]. *Arabidopsis* mutant lines overexpressing rice full-length cDNA library under the control of CaMV 35S promoter facilitated functional analysis of rice genes [30]. Several genes conferring 506 altered phenotypes, such as early/late heading, short/long leaf, narrow/wide leaf, low-tillering, etc., were identified from the screening of a large population of transgenic rice overexpressing full-length cDNAs of rice [31]. Very recently, functional screening of *Arabidopsis* transgenics overexpressing FL-cDNA library of soybean led to identification of favorable genes, such as those involved in promoting plant growth, drought tolerance, increased seed size, etc. [32]. Recently, FOX/COX-based gene hunting system has been used in yeast to identify candidate genes involved in salt stress tolerance in halophytes, such as *Zoysia matrella* [33], *Paspalum vaginatum* [34], *Atriplex canescens* [35], and *Ipomoea pes-caprae* [36]. Yet, constitutive activation of stress regulatory genes can disturb cell proliferation and development, resulting in abnormal phenotypes, such as dwarf and sterile plants [37,38]. To resolve this issue, a controlled cDNA overexpression system (COS) by Gateway™ cloning of cDNA library was reported for the identification of salt tolerance genes in *Arabidopsis* [39].

Here, we report on the construction of an optimized cDNA overexpression system for the wild rice halophyte *P. coarctata* that confers dominant stress-tolerance phenotypes in heterologous *Arabidopsis* model system, and demonstrate that this system can be exploited as a simple and versatile genetic tool for primary screening of genes in non-model extremophiles for identification of regulators of stress responses.

2. Materials and Methods

2.1. Plant Materials

Vegetative cuttings of *Porteresia coarctata* (*Oryza coarctata*; Acc. No. IRGC 104502; IRRI, Philippines) containing rhizomes, roots and culms with two to three leafs were grown in 4 L plastic pots filled with garden soil: potting mix (1:1) and irrigated with tap water until establishment inside a greenhouse under 12 h light and 29/22 °C day/night temperature regime. Young uniform plants with three to four leaves, rhizome and roots were transferred to 4 in sand-filled plastic pots with bottom drainage holes (one plant/pot) and placed in large plastic trays with 10 L Hoagland's nutrient solution that was replenished every ten days. After 45 days, the solution was replaced with fresh nutrient solution mixed with commercial synthetic sea salt mix (Aquarium Systems, Mentor, OH) at 25 parts per thousand to impose salt stress. Leaf and root tissues were harvested in liquid N_2 from unstressed control (0 h), and at 24 h, 48 h, 72 h, 1 week and 2 weeks after stress and stored at −80 °C for RNA extraction.

2.2. RNA extraction and generation of full length cDNA library

Total RNA was extracted from 100 mg of leaf and root tissues using Trizol™ (Invitrogen, Carlsbad, CA) followed by treatment with DNase I (Qiagen, Valencia, CA). The RNA integrity and quality were assessed by running a 2 µl aliquot in an 1.2% (w/v) agarose/formaldehyde gel as described earlier [40]. The RNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

RNA was treated with RiboMinus Plant Kit reagents (ThermoFisher Scientific Inc, Waltham, MA) to selectively deplete ribosomal RNA in the library. RNA from the control and stressed leaf and root tissues at different time points in three biological replicates were pooled in equimolar concentration and reverse-transcribed using SMARTer (switching mechanism at 5' end of RNA transcript) PCR cDNA synthesis kit (Clontech Laboratories Inc, Paolo Alto, CA) according to the user manual. Briefly, 1 µg of pooled RNA was annealed with 1 µl of 12 µM 3′ SMART CDS primer in a total volume of 4.5 µl at 72 °C for 2 min followed by reverse transcription with 2 µl of 5X first-strand buffer, 0.25 µl of 100 mM DTT, 1 µl of 10 mM dNTP mix, 1 µl 12 µM SMARTer IIA oligonucleotide, 0.25 µl of RNase inhibitor and 1 µl of 100 U/ml SMARTScribe reverse transcriptase at 42 °C for 1 h in a Bio-rad T100 ™ thermal cycler (Bio-rad, Hercules, CA). Long-distance PCR was performed with 1 µl 1st strand cDNA reaction mixture, 10 µl of 10x Advantage 2 polymerase buffer (Clontech), 2 µl of 10 mM dNTP mix, 2 µl of 12 µM 5′ PCR primer and 2 µl 50x Advantage 2 polymerase mix (Clontech Laboratories) using the thermal profile: 1 cycle at 95 °C for 1 min, 18 cycles at 95 °C for 15 s, 65 °C for 30 s, and 68 °C for 6 min.

2.3. Construction of FL-cDNA library and plant transformation vector

A schematic diagram of the construction of plant transformation vector is depicted in Fig. 1. To construct a Gateway-compatible FL-cDNA library, 10 µl aliquot of Advantage 2 PCR product was used as template for five cycles of amplification with ATTSM1 and ATTSM2 primers (Supplementary Table S1, [39]). The cDNA was normalized to reduce representation of highly abundant genes following the method described by Zhou et al. [41]. The purified cDNA PCR product

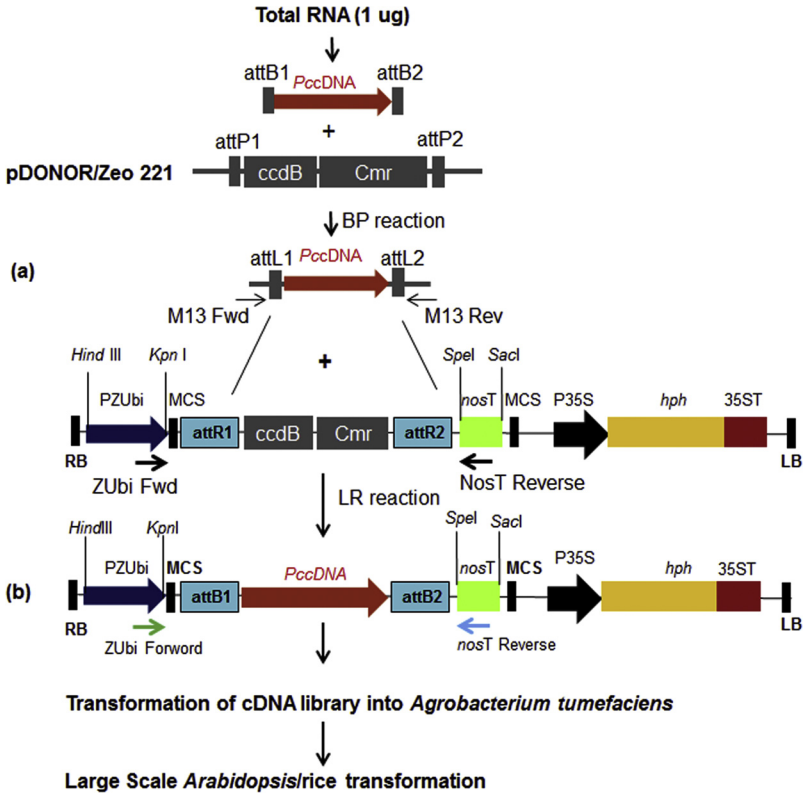

Fig. 1. Strategy for generating the overexpression library carrying *Porteresia coarctata* cDNAs for generating transgenic *Arabidopsis* and rice plants. a) Primary cDNA library in pDONOR/Zeo 221 (pENTR vector); b) cDNA library in pMDC99 + PZUbi-cDNA-nosT (destination binary vector).

(QIAquick PCR purification kit, Qiagen) was cloned into pDONOR 221/Zeo using BP clonase reaction kit (Invitrogen). Aliquots of the reaction mix were transformed into chemically competent *E. coli* DH5α cells. The quality of the library with cDNA inserts was checked by PCR-amplification of 48 randomly selected transformant colonies with M13 primers (Supplementary Table S1).

First, the 250-bp nosT was PCR-amplified from the pCAMBIA1301 with Phusion polymerase (New England Biolab, Ipswich, MA) using forward and reverse primers with *Spe*I and *Sac*I restriction endonuclease (RE) recognition sites (Supplementary Table S1), respectively. The PCR fragment and the binary vector pMDC99 [42] were both digested with same restriction enzymes (REs) and ligated to generate pMDC99-nosT. The Ubi promoter of pUC18-PZubi [43] was PCR amplified with Phusion polymerase using forward and reverse primers with *Hind*III and *Kpn*I RE sites, respectively, and cloned into the corresponding sites of pMDC99-nosT to yield pMDC99-PZubi-nosT (Fig. 1). The sequence and orientation of promoter and terminator was confirmed by restriction digestion analysis and sequencing. The cDNA library was cloned, in 40 independent reactions with 150 ng of the cDNA in each reaction, into Gateway-compatible plant transformation binary vector, pMDC99-PZubi-nosT with the maize ubiquitin (Ubi1) gene promoter region at *Hind* III and *Kpn* I sites located upstream of attR1site, and nopaline synthase terminator (nosT) region at the *Spe*I and *Sac*I sites located downstream of attR2 site using the LR clonase reaction kit (Invitrogen). The resulting pMDC99-PZubi-FL-cDNAs-nosT library were transformed into *E. coli* DH5α. Colonies (˜220,000) were pooled from all plates and plasmid DNA isolated from this library was transformed in aliquots of 1 µg into *Agrobacterium tumefaciens* strain LBA4404/pSB1 by freeze-thaw method as described earlier [44]. All *Agrobacterium* colonies obtained (˜300,000; 10 batches of 30,000 colonies in 10 ml each) were pooled and suspended in 100 ml YEP medium for subsequent use in transformation.

2.4. *Arabidopsis* in-planta transformation and screening of transgenic plants for salinity tolerance

The pMDC99-PUbi-cDNAs-nosT library was introduced into *Arabidopsis thaliana* ecotype Columbia by *Agrobacterium tumefaciens*-mediated in-planta transformation by floral dip method as described earlier [45]. All seeds of infiltrated plants were collected in bulk and selected for dominant gain-of-function salt tolerance phenotype initially through a germination assay. Seeds were germinated on plates with ½ MS medium [46] supplemented with 15 mg/L hygromycin and 150 mM NaCl. Forty seven seedlings that formed true leaves under salt after two weeks were transferred to fresh half-strength MS basal medium with NaCl but no hygromycin for one more week before transferring to pots filled with Miracle-Gro™ potting mix to set seeds. Forty four out of 47 plants completed the life cycle and set the seeds. T_1 seeds obtained from 44 plants were further screened for salt stress tolerance on ½ MS medium supplemented with 150 mM NaCl and hygromycin for two weeks. Wild type (WT) *Arabidopsis* seeds were germinated on ½ MS basal media without salt and hygromycin. Forty-one transgenic lines survived salt stress and were transferred to pots along with WT. Transgenic lines and WT plants after a week of establishment in pots were irrigated with 10% Hoagland's nutrient solution containing 150 mM NaCl for three weeks. T_2 seeds were collected from the surviving plants for further analysis.

Five week-old selected T_2 homozygous *Arabidopsis* transgenics exhibiting dominant salt stress tolerance phenotype with ectopic expression of cDNAs (Table 1) and WT plants were irrigated with 10% nutrient solution salinized with 200 mM NaCl. Rosette leaves harvested after three weeks of stress were used for analysis of physiological traits and gene expression. After three weeks, plants were transferred to normal 10% Hoagland's nutrient solution for observation on recovery.

Table 1
Analysis of cDNA inserts rescued from the salt tolerant T_0 transgenic *Arabidopsis* plants obtained by transformation with *Agrobacterium tumefaciences* harboring the *Porteresia coarctata* cDNA library

Arabidopsis line #	Similarity to Gene	Length (bp)	Full/ partial	GO ID	E value	Reference
1	Nucleoside diphosphate kinase1	425	partial	GO:50096950	9.00E-126	AY649743.1
4,27	Methallothionein-like protein (MT2)	652	full length	GO:61191785	7.00E-127	AY833009.1
6	Similar to serrate RNA effector molecule	407	partial	GO:1002276161	7.00E-118	XM_015786944
10,21,35,43	Methallothionein-like protein (MT2B)	646	full length	GO:385718847	3.00E-112	JF969225.1
11U, 17	Ribosomal L23 protein	772	Full length	GO:1002265453	0.00E+00	XM_015781570.1
11L	Soluble inorganic pyrophosphatase 4	445	partial	GO:1002231405	8.00E-80	XM_001540144
12	Metallothionein MT3	789	full length	GO:119393774	6.00E-110	EF136378
13, 19	60S ribosomal protein L18a	840	full length	GO:1002266740	0.00E+00	XM_015782215
18U, 24	UTP:RNA uridylyltransferase 1	713	partial	GO:1002869510	0.00E+00	XM_015841452
20	Serine/arginine-rich splicing factor SR45a	591	partial	GO:1002257134	5.00E-154	XM_015777396
22	*Oryza sativa* Japonica Group chromosome 8	500	full length	GO:42407954	4.00E-58	AP004015.3
26	Photosystem II 10 kDa	688	full length	GO:115470574	3.00E-174	NM_001065421
28,41	*Oryza coarctata* chloroplastidic AtpF	776	partial	GO:281312580	0.00E+00	FJ908139.1
31	Haloacid dehalogenase-like hydrolase domain-containing protein	742	partial	GO:1002299076	5.00E-148	XM_015757457
36	Polyubiquitin (Rubq1)	763	partial	GO:701448701	0.00E+00	KJ909980.1
37	Polyadenylate-binding protein-interacting protein 7	806	partial	GO:573947112	0.00E+00	XM_006655822.1
38	40S ribosomal protein S20-1 (S22)	710	full length	GO:1002302870	0.00E+00	XM 015759332
40, 42, 47	Vacuolar protein sorting-associated protein 2.1	819	partial	GO:1002303433	2.00E-161	XM_015759617
3, 9, 14,15, 18 L,23,25, 44, 45, 46	*Aplysia puncatata* cyplasinS (ek435 gene)	509	partial	GO:11967688	2e-131	AJ30480.1

2.5. Physiological phenotyping of homozygous transgenic plants under salt stress

Five *Arabidopsis* homozygous independent transgenic lines and respective WT were germinated on ½ MS basal medium. Ten two-week-old plants from each transgenic *Arabidopsis* line were transferred to ½ MS supplemented with 200 mM NaCl and root and shoot length was recorded after a week under salt stress. Ten plants of *Arabidopsis* were transferred to pots. One-month-old plants were subjected to salt stress by irrigation with Hoagland solution supplemented with 150 mM NaCl for 10 days. The salt tolerance phenotype and physiological responses were recorded for transgenics and WT plants at day 0 and 3. After a week of stress, all the plants were transferred to fresh nutrient solution without salt for recovery.

2.5.1. Relative water content and Membrane stability index

Equal amounts of fully expanded leaves from both WT and different transgenic lines were collected from control and 3 day salt stress treatments to estimate both relative water content (RWC) and membrane stability indexes (MSI) following the method described earlier [44,45].

2.5.2. In vivo localization of O_2- and H_2O_2

Accumulation of reactive oxygen species (ROS) in transgenic and WT under stress and control conditions was observed by nitro-blue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) assays following the method described in Sengupta et al. [47]. Briefly, in vivo detection of O_2^- was done by immersing the leaf samples in NBT solution (10 mM Na_2HPO_4; pH 7.8) at room temperature for 2 h and then exposed to light for 12 h until blue spots appeared. For the detection of H_2O_2, leaves were incubated in DAB solution (20 mM Tris-HCl, pH 3.8) at room temperature for 8 h in dark, and then leaf samples were exposed to light until leaf samples turned into brown in color. Leaf samples were immersed in absolute ethanol for bleaching chlorophyll contents before visualization and photography.

2.6. Rescue of cDNA inserts from salt tolerant transgenic plants

The cDNAs of the T-DNA insertions were rescued from the salt tolerant *Arabidopsis* plants by PCR amplification of 50 ng of genomic DNA using ZUbi-fwd and nosT-rev primers (Supplementary Table S1). Purified DNA fragments (QIAquick gel extraction kit, Qiagen) were single-pass sequenced using the ZUbi-seq primer (Supplementary Table S1) on an ABI3130XL genetic analyzer (Applied Biosystems, Hercules, CA). The sequences were searched for sequence similarity against the non-redundant DNA and protein databases using BlastN/BlastX (www.ncbi.nih.gov/BLAST).

2.7. Expression analysis of inserted cDNAs

Total RNA was extracted from 100 mg leaf tissues collected from control and three-week salt-stressed transgenic plants and WT plants using Trizol™ as per the manufacturer's instructions (Invitrogen). One µg of total RNA was reverse transcribed using a iScript cDNA synthesis kit (Biorad). Semi-quantitative RT-PCR was performed with 2 ul of diluted (1:10) 1st strand cDNA using the method described earlier [44]. The elongation factor genes from *Arabidopsis* (*AtEF1a*) were used as the internal controls for the template validation (Supplementary Table S1).

3. Results

3.1. Transformation, Salt stress tolerance assay of T_0 transgenics of *Arabidopsis thaliana*

PCR analysis of 48 randomly selected *E. coli* colonies carrying the pMDC99-PZubi-cDNAs-nosT library with M13 F/R primers showed cDNAs inserts ranging from 0.5 to 2.5-kb, with an average of 0.65-kb (Supplementary Fig. S1).

All T_0 (~10^7) seeds pooled from the *Agro*-infiltrated *Arabidopsis* plants were screened for salt tolerance with 150 mM NaCl under hygromycin selection. After three weeks, 47 T_0 seedlings produced true leaves and well-developed roots (Fig. 2a) that were transferred to fresh ½ MS medium for two more weeks. Forty four plants recovered well and produced seeds upon transfer to pots. On the other hand, WT seeds showed late germination and produced seedlings with either no leaves or bleached yellowish leaves that subsequently died under salt (Fig. 2a) in comparison to unstressed control (Fig. 2a). Germination of T_1 seeds derived from the 44 T_0 plants on ½ MS medium containing 150 mM NaCl and hygromycin showed 41 lines with heathy seedlings (Fig. 2b), which were subsequently transferred to pots to advance to T_2 generation. Three lines, P#32, P#33, and P#33, were escapes as the seeds failed to germinate under selection.

PCR analysis showed that all salt tolerant *Arabidopsis* transgenic lines had one cDNA insert, except three lines (#11, #18, and #26) that

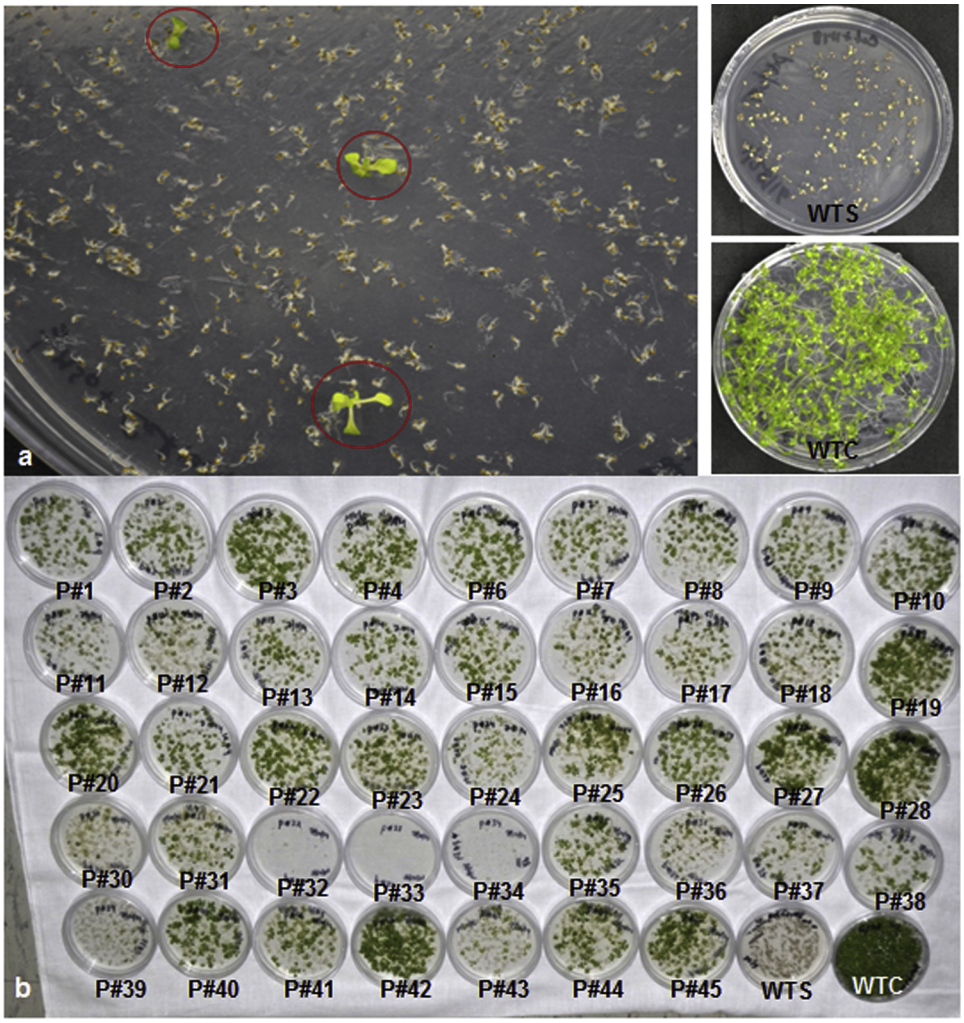

Fig. 2. Screening of *Arabidopsis* transgenics expressing cDNA library of *Porteresia coarctata* for salt tolerance using seed germination assay in MS medium supplemented with 150 mM NaCl. a = T_0 seeds; b = T_1 seeds; WTC = wild type Colombia under control; WTS = wild type Colombia seeds under salt stress; WT seeds without salt.

had two inserts, with insert size 0.3–1.9 kb (Supplementary Fig. S2). Similarity search revealed that 27 lines had cDNA inserts that had match to sequences related to plants (Table 1). The results showed that 19 out of 27 transgenics were independent with a unique cDNA insert, and 14 were full-length cDNAs whereas five were partial sequences but with at least one complete open reading frame. Interestingly and as yet unexplained, 10 lines contained a cDNA that showed similarity with a cytotoxic cyplasin gene of sea hare (*Aplysia punctate*), a species of sea slug.

3.2. Salt stress tolerance assay of T_1 and T_2 Arabidopsis transgenic plants

Forty one T_1 transgenic lines that showed salt tolerance on ½ MS with 150 mM of NaCl were grown in pots. After five weeks, pots were saturated with 10% Hoagland's nutrient solution containing 150 mM of NaCl until maturity. Of these, line #4 and 27 (metallothionein 2, MT2), line #10 (metallothionein 2B, MT2B), line #11(ribosomal gene 23, L23a and inorganic pyrophosphatase, PP), line #12 (metallothioinein 3, MT3), line #17 (ribosomal gene 23, L23a), line #19 (ribosomal gene 18, L18a), line# 26 (photosystem II 10 kDa, PSII-10 kDa), Line# 27 (MT), line #31 (haloacid dehalogenase-like hydrolase, HAD), and line #40 (vacuolar protein sorting-associated protein 2.1,VPS2.1) showed high tolerance to salinity when compared to other lines and the WT. The transgenic plants showed less leaf yellowing, withering and wilting phenotypes compared to the WT (Fig. 3a,b). A variation in the phenotype was observed among transgenic lines expressing different isoforms of metallothionein genes. Lines #4, #10 and #12 maintained better vegetative growth than line #27, which was shorter and late in flowering (Fig. 3a). Line #31 was also shorter in height and late in flowering in comparison to WT and other transgenics under non-stressed control conditions (Fig. 3a). Although 10 transgenic lines showed good vegetative response under salinity, only lines #10, #19, #26, #27, and #40 were able to produce fertile siliques with seeds, whereas the WT died at the vegetative stage (Fig. 3c). Germination test of T_2 seeds of these five transgenic lines overexpressing different genes under hygromycin selection showed homozygosity of the lines. One-week-old homozygous seedlings of each line were transferred to ½ MS_0 medium supplemented with 200 mM NaCl and allowed to grow vertically for three weeks. The transgenics lines were healthy and showed enhanced root and shoot growth compared to WT under salt stress (Fig. 4a,b). Transgenic *Arabidopsis* lines maintained higher relative water content (RWC) than the WT plants under salt stress (Fig. 4c). Similarly, the membrane stability index (MSI) of transgenic lines was higher, which indicated transgenics had reduced electrolyte leakage compared to WT plants under salt stress (Fig. 4d).

In vivo localization studies revealed that the leaves of WT plants showed more accumulation of reactive oxygen species with higher O_2- (Fig. 4e) and H_2O_2 (Fig. 4f) content compared with transgenic lines under salt stress, as shown by the blue and deep brown coloration of the leaves. RT-PCR analysis of transgenic and WT plants showed increased

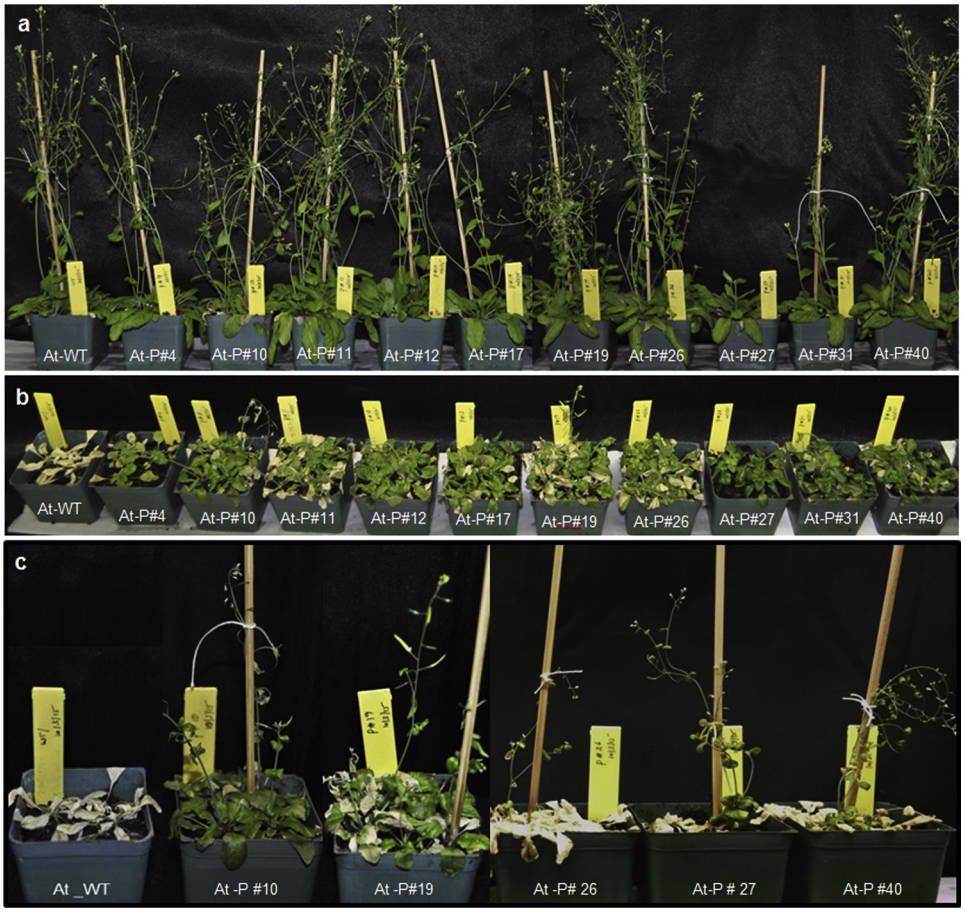

Fig. 3. Phenotype of *Arabidopsis* transgenics overexpressing *Porteresia coarctata* cDNAs under control (a) and under salt (150 mM) stress (b). Only five transgenics were able to complete the life cycle by producing fertile siliques upon recovery from salt stress (c).

accumulation of corresponding transcripts in transgenics under salt stress compared to control, whereas, as expected, gene-specific mRNA was detected in the WT (Supplementary Fig. S3).

4. Discussion

Halophytes, as wild crop-relatives, have been studied as important non-crop models for both basic and translational research to demonstrate differences in molecular mechanisms underlying their salinity tolerance [48–51]. Except for a few recent studies [17,50], the halophytes that have been studied extensively are dicots. Keeping in view the anatomical and physiological differences between monocot and dicot halophytes in their adaptive response to salinity, *Spartina alterniflora* and *Porteresia coarctata* were proposed as monocot halophyte models for improvement of salt stress tolerance in rice [2]. A single-gene overexpression strategy has been used to validate function of salt stress-responsive genes from the grass halophyte *Spartina alterniflora* using rice and *Arabidopsis* models [44,45,47,52]. The cDNA over expression system (COX) is a simple and powerful technology to validate functions of one to several genes associated with the regulation of specific stress responses, yet this has not been used in either of the above two grass halophytes. In this report, through the direct gain-of-function phenotypes of *Arabidopsis* plants overexpressing *P. coarctata* cDNA library under the control of a strong constitutive maize ubiquitin promoter through COX strategy, we identified specific genes with positive involvement in salt tolerance mechanisms.

The *P. coarctata* cDNAs represented in the present study had sizes ranging from 0.5 to 2.5 kb with an average of 0.65-kb (Supplementary Fig. S1), which is different from a previously reported COS library [39]. However, the average sizes of the inserts observed in our study are similar to the sizes of the transcripts reported in *P. coarctata* [17] and rice cv. Pokkali transcriptome [53]. Ninety two percent (34/37) of the salt tolerant *Arabidopsis* transgenics contained one functional cDNA insert of which only ˜48% (10/21) were full-length transcripts. This could be due to the fact that longer transcripts often get truncated due to strong size bias against large fragments during cDNA synthesis, cloning, ligation and bacterial transformation [54]. Another, probable cause is the preferential cDNA insertion during construction of the *Agrobacterium tumefaciens* library, which is evident from the high frequency of a particular cDNA in our transgenic lines. Also, the growth rates of *Agrobacterium* populations harboring a variety of cDNAs may not be synchronized. Another possibility may be that strong ectopic overexpression of various cDNAs, especially transcription factors or signal transducers, under pUbi promoter had deleterious/lethal effect on the transgenic cells/plants.

High throughput screening for salt tolerance permitted identification of genes, which upon overexpression, could enhance the germination rate, increase seedling survival and/or complete reproductive cycle under salinity. For example, germination under salt stress followed by enhanced growth of transgenic *Arabidopsis* line #10 (*MT2B*), line #27 (*MT2*), and rice line #6 (*MT2*) compared to WT is governed by the gene family that encodes detoxification enzymes and related proteins, metallothioneins (MTs). Plant MTs are low-molecular weight (4–8 kDa), cysteine-rich metal chelators with ability to detoxify heavy metals by buffering cytosolic metal concentrations [55]. In addition to their function via spatial expression as metal chelators, the role of plant MTs in abiotic stresses, such as salt, oxidative, and dehydration stresses as well as in senescence and hormonal alterations are well established

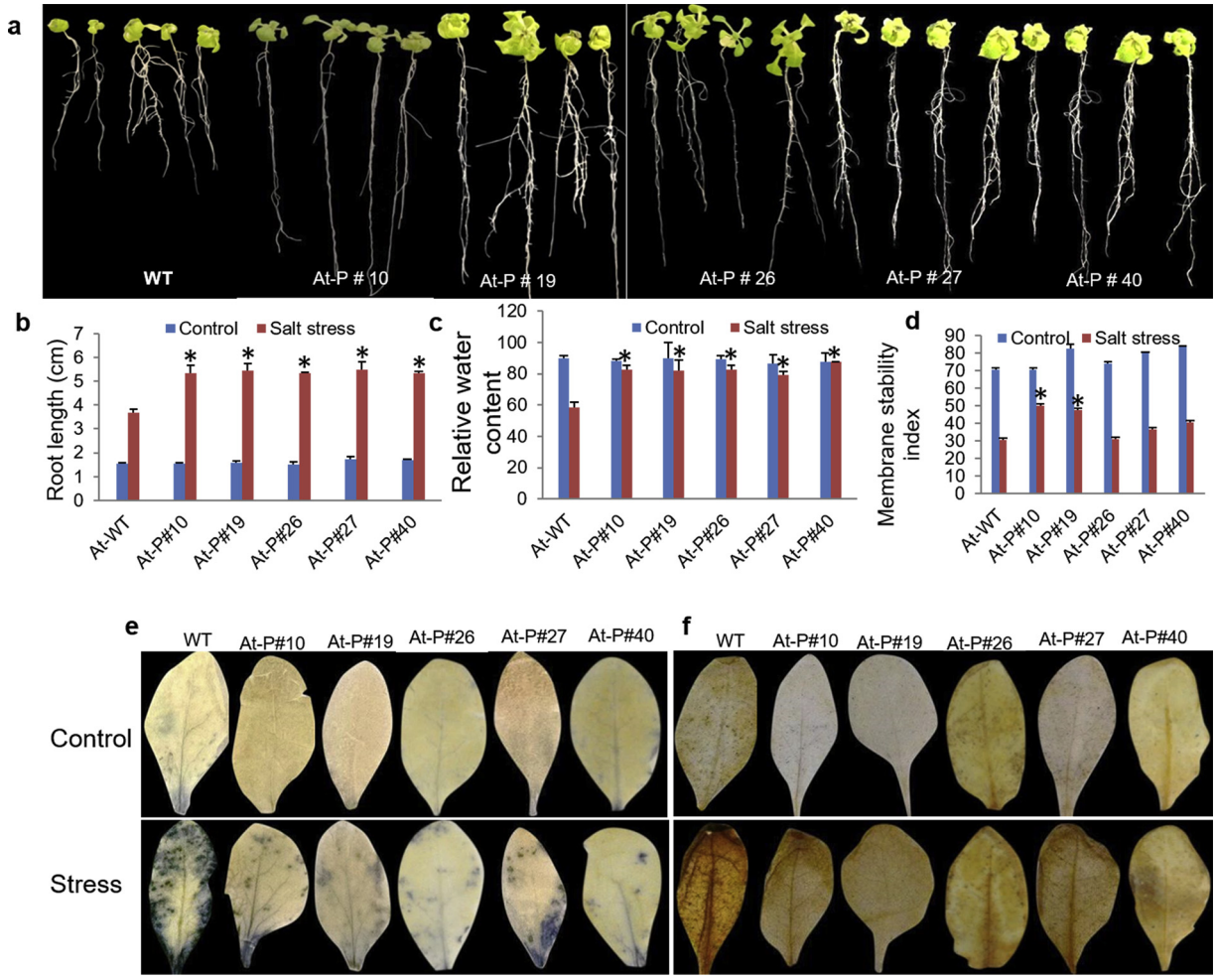

Fig. 4. Salt tolerance phenotype of *Arabidopsis* transgenic plants overexpressing *Porteresia coarctata* cDNAs that show better shoot and root growth than the WT under 150 mM NaCl (a, b). The transgenics maintained higher relative water content and membrane stability (c, d) and lower accumulation of reactive oxygen species (e,f) under salinity. Bars with * represent values that are significantly different under stress ($P < 0.005$; $n = 3$).

[56–59]. Expression of *OsMT1e-P*, a type 1 MT isolated from a salt tolerant rice 'Pokkali', was highly responsive to seedling stage salinity stress, and its ectopic expression provided transgenic tobacco plants with tolerance against salinity, drought, cold, heat, and heavy metals (Cu^{2+} and Zn^{2+}) via toxic ROS scavenging [60]. Transgenic tobacco lines expressing a type 2 MT, *SbMT2* from *Suaeda breviligulata* manifested significantly enhanced salt and drought tolerance [61]. Ectopic expression of *Brassica juncea BjMT2* gene conferred transgenic *Arabidopsis* plants with increased percentage seed germination, fresh weight, dry weight, and chlorophyll content under Cu and Cd stress [57]. In the present study, *Arabidopsis* lines #10 and #26 showed higher germination and consequently better shoot and root growth compared to WT (Fig. 1,2). *MT2*-overexpressing transgenic *Arabidopsis* were shorter as compared to the WT (Fig. 2). Short height phenotype was observed in transgenic rice plants overexpressing *OsMT2b* [62], which could be due to its possible effect on endogenous cytokinins that are involved in root development and seed embryo germination. Abiotic stress perturbs metabolic balance of the cell, resulting in excessive production and accumulation of ROS, which in turn damage cell membranes, nucleic acids, and chloroplast pigments and thus disrupt cellular functions and ultimately lead to cell death [63,64]. Role of MTs in ROS scavenging/detoxification and maintenance of cellular homeostasis under stress conditions was evident with less H_2O_2 and O^{2-} accumulation in the transgenics overexpressing MT-2 and MT2b. The transgenic lines retained higher RWC than WT (Fig. 4). Further, MT-2 plays a role in counteracting the osmotic shock, thus protecting the membrane of transgenics with higher membrane stability and less electrolyte leakage under salt stress (Fig. 4).

Accumulation of higher amounts of small (40S) and large (60S) subunits of ribosomal proteins, that are major components of ribosomal complex, is common to a plant's general response to external stimuli. Many ribosomal proteins participate in specific plant processes or developmental stages [65], and are induced in response to phytohormones [66,67] and stressors, including salt [40], heat [68], cold [69], UVB [70], and phosphate or iron deficiency [71]. In addition, studies imply indirectly that 60S L18a and L23a, and 40S S18a genes are involved in abiotic stress response of plants [40,72,17]. In agreement with these observations, our results showed that overexpression of S22, L18a, and L23a conferred salt stress tolerance in transgenic *Arabidopsis*.

Stressors reduce light-absorbing efficiency of both photosystems (PSI and PSII) and hence affect photosynthetic capacity [73,74]. PS II is relatively more sensitive to salt stress [75]. Earlier studies reported an upregulation of a PSII 10 kDa protein under drought [76] and salt stress [71], which suggests that this protein, as a key component of water-splitting complex, may protect the PS II machinery from drought and salinity induced ROS. Consistent with the previous reports, the gene for PSII 10 kDa protein was upregulated under salt in the transgenic *Arabidopsis* line #26, which showed better physiological performance compared to WT under salt stress (Fig. 4).

Ectopic expression of vacuolar protein sorting-associated protein (VPS2.1) in *Arabidopsis* line #40 showed enhanced salt tolerance

(Fig. 4). Increase in cellular salt concentration in plants through endocytic uptake under high salinity leads to an increase in intravesicular ROS that act as secondary messengers in salt stress-elicited signaling pathways [77,78]. Plants use endosomal complex required for transport (ESCRT-0, I, II, III) components for biogenesis of multi-vesicular body (MVB) system. ESCRT-III complex consists of four subunits in two different sub-complexes, Vps20-Snf7 core sub-complex and Vps2-Vps24 coat sub-complexes [79] along with two associated proteins VPS60 and VPS46 that activate plasma membrane AAA ATPase, VPS4/Suppressor of K + transporter growth defect (SKD1), that dissemble ESCRT-III complex components. Upregulation of various VPS proteins under salt stress [80] and boron-toxicity [81] has been reported. Plants with mutations in the ESCRT-II component *VPS22* or ESCRTIII components *VPS20* and *SNF7* (sucrose non-fermenting 7) exhibited mild salt sensitivity [82]. Similarly, loss of VPS9a function in plants affected their ability to form a vacuolar system in the inner layers, and made the plants extremely sensitive to salinity [83].

In summary, the present study demonstrated that COX strategy could be successfully used as an alternative of multicopy suppressor system and/or single cDNA overexpression strategy to facilitate high throughput, systematic and genome-wide functional analysis of natural variants of genes from a wild rice halophyte for salt tolerance in *Arabidopsis* model. Except for the ribosomal protein L18a that showed 100% similarity between *P. coarctata* and *Arabidopsis thaliana*, other genes showed 40-70% similarity. Similarly, comparison of the genes of *P. coarctata* and *Oryza sativa* showed at least 10-20% sequence difference between the two species (Supplementary data 1). Thus, the salt-responsive genes identified from *Porteresia coarctata* were expected to show similar results in rice. Indeed, preliminary results indicated that transgenic rice plants overexpressing a few genes manifested enhanced salt stress tolerance (Supplementary Figure S4). Such structural differences have been reasoned for the superiority of the halophyte genes in conferring resistance in plants against abiotic stresses [47]. Nonetheless, gene function need to be validated by further characterization of the gene(s) through knock-down strategies via inducible RNAi, transcriptome profiling and/or chromatin immunoprecipitation [39]. Also, the responses of *Arabidopsis* plants overexpressing its native genes orthologous to those identified in *P. coarctata* in this study will confirm the superiority of the halophyte genes. Constitutive high activity of genes under ubiquitin promoter, as used here, could sometimes be toxic, thus conferring critical and lethal effects on the growth of plants, which reduced the sum of FL-cDNAs integrated into the *Arabidopsis* genome. This problem can be circumvented by inducible (controlled) overexpression of genes under suitable stress-inducible or tissue-specific promoters. Further, multiple individual libraries with different insert sizes need to be prepared and mixed for transformation to maximize the representation of the donor genes. Normalization of screening procedure, such as screening for tolerance at germination, seedling and/or reproductive stage independently could lead to identification of more number of genes, including known genes, implicated in salt stress response at different stages of growth and development. Future research will focus on elucidation of mechanisms underlying the biological implications of identified genes, including the partial genes with a functional open reading frame, in salt stress tolerance of cereal crops, such as rice.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cpb.2019.100107.

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