

Isolation of genes conferring salt tolerance from *Piriformospora indica* by random overexpression in *Escherichia coli*

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Abstract *Piriformospora indica*, a root endophytic fungus identified in the Indian Thar desert, colonizes the roots of plants and provides resistance towards biotic stress as well as tolerance to abiotic stress in the plants. Despite its positive impact on the host, little is known about the *P. indica* genes that are involved in salt stress tolerance. Therefore this study was conducted to identify and isolate high salinity-tolerance genes from *P. indica*. Thirty-six salinity-tolerance genes were obtained by functional screening, based on random over expression of a *P. indica* cDNA library in *Escherichia coli* grown on medium supplemented with 0.6 M NaCl. The salinity tolerance conferred by these 36 genes in bacteria was further confirmed by using another strain of *E. coli* (DH5 α) transformants. However when the expression of these 36 genes was analysed in *P. indica* using quantitative RT-PCR, we found only six genes were up-regulated by salt stress. These six genes are involved in different cellular processes,

such as metabolism, energy and biosynthetic processes, DNA repair, regulation of protein turnover, transport and salt stress tolerance. This work presents the basis for further molecular analyses of the mechanisms of salt tolerance in *P. indica* and for the use of this endophyte to confer salt tolerance to plants.

Keywords *Escherichia coli* · Functional screening · *Piriformospora indica* · Root endophytic fungus · Salt tolerance conferring genes

Introduction

Despite a worldwide intensification of agriculture and tremendous progress towards increasing yields in major crops over the last decades, the goal to reduce the problems associated with hunger is far from being reached (Mahajan and Tuteja 2005). Major causes for crop losses are abiotic and biotic stresses, which are due to unfavorable climate and soil conditions, plant diseases and pests. A key to this is to increase knowledge of the intricate and dynamic communications between plants and their interacting beneficial microbial partners. The benefits in mycorrhizal associations arise from the nutrient transport between the plant roots and fungal hyphae (Bonfante and Genre 2010). The carbon source is transported from the plant to the fungus, whereas fungal hyphae serve as a fine link between the roots and the rhizosphere and improve the supply of the plant with inorganic nutrients (Harrison 1999; Harrison et al. 2002; Bücking and Heyser 2003; Karandashov et al. 2004; Yadav et al. 2010). Although the importance of arbuscular mycorrhizal associations was recognized a long time ago, the knowledge about the mechanisms leading to

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the establishment and functioning of this symbiosis is still limited (Bonfante and Genre 2010; Limpens and Bisseling 2003; Breuninger and Requena 2004; Marx 2004; Parniske 2004).

Soil salinity has become a severe global problem, with 950 Mha of the earth's soil being saline (Li et al. 2005). Therefore methods to improve the stress tolerance and survival of plant seedlings have become more and more important for crop production, reforestation and ecosystem reestablishment in saline arid and semiarid areas. Excessive amount of ions present in soil cause injury to plant roots, water deficit, ion toxicity, nutrient deficiency and damage to metabolism, ultimately resulting in cell death, stunted growth and decrease in agricultural production (Shannon 1997).

One of the ways to evoke salinity-stress tolerance in crops is by the exploitation of endophytic arbuscular mycorrhizal fungi (AMF), which live in reciprocally beneficial relationships with about 80 % of land plants (Newman and Reddell 1987). Furthermore, the symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Waller et al. 2005). *Piriformospora indica* is a root-colonizing basidiomycete of the order Sebaciniales (Varma et al. 1999; Weiss et al. 2004; Selosse et al. 2007). It has been reported that *P. indica* colonizes roots and increases the biomass of both monocot and dicot plants (Varma et al. 1999; Kumar et al. 2009). In contrast to AMF, *P. indica* can be easily grown on synthetic media allowing for large-scale propagation and a possible use in agriculture for crop yield improvement (Pham et al. 2004). Hosts include the cereal crops rice, wheat, and barley as well as many Dicotyledoneae, including *Arabidopsis thaliana* (Varma et al. 1999; Peskan-Berghofer et al. 2004). The growth-promoting activity of the fungus resulted in enhanced barley grain yield, imparted tolerance to abiotic stress such as salt stress, and conferred resistance to root and leaf pathogens like the necrotrophic fungi *Fusarium culmorum* (root rot), *Fusarium verticillioides* and the biotrophic fungus *Blumeria graminis* (Kumar et al. 2009; Waller et al. 2005). Recently we found that *P. indica* helps the host plant during high salt stress (Jogawat et al. 2013). *P. indica* colonization of rice plants resulted in an increase in root and shoot lengths, dry weight, total chlorophyll contents as compared to the non-colonized plants (Jogawat et al. 2013). At the cellular level, adaptive mechanisms are conserved across genera and have an important role to play during salt stress, therefore, it is assumed that fungal stress genes can be screened functionally by their random over expression in a model organism. Several reports are available on functional screening of the plant stress genes by their random over-expression in yeast and *E. coli* (Joshi et al. 2009; Kanhonou

et al. 2001; Forment et al. 2002; Rausell et al. 2003; Mundree et al. 2000; Yamada et al. 2002, 2003). The present work aimed to identify and isolate genes of *P. indica* which putatively impart salt stress tolerance. For this purpose, a *P. indica* cDNA library was randomly overexpressed in *E. coli*. The salinity tolerance of the putative identified genes was checked in another strain of *E. coli* (DH5 α) transformants. Expression of these genes was analysed in *P. indica* using quantitative RT-PCR.

Materials and methods

Fungus materials, growth conditions and stress treatments

This experiment was conducted to observe the influence of different salt (NaCl) concentrations on fungal biomass to determine if *P. indica* [Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSM 11827)] can resist high salt concentrations. For broth batch culture of *P. indica*, the mycelium of 4–5 fully grown fungal agar discs (4 mm in diameter) was inoculated into 500 ml Erlenmeyer flasks containing 250 ml of Hill and Kaefer broth medium (Hill and Käfer 2001). It was incubated at 30 ± 2 °C with constant shaking at 150 rev/min in a metabolic shaker (Multitron, Incubator, HT-Infors, Switzerland). *P. indica* was subjected to different concentration of salt (NaCl): 0, 200, 400, 600, and 800 mM, to check its ability to grow at high salt concentration. Growth of *P. indica* was measured in terms of dry weight by collecting fungal mycelium at 0, 5, 10, 15 and 20 days as described previously (Kumar et al. 2009).

Construction of the cDNA library and functional screening of salinity tolerant genes

To make the cDNA library, RNA was isolated from the *P. indica* grown in 0.4 M NaCl as follows. Fungal mycelia were filtered through Whatman filter paper no. 1 and then wrapped in aluminium foil and kept in liquid nitrogen. Frozen mycelia were homogenized in 1 ml of TRIzol[®] reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) reagent with the help of mortar and pestle in liquid nitrogen. The homogenized sample was transferred to a 2-ml microcentrifuge tube and was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform per 1 ml of TRIzol reagent was added, vortexed for 15 s and was incubated at 15–30 °C for 2–3 min. The sample was centrifuged at 12,000 \times g for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml

isopropyl alcohol. The sample was incubated at room temperature (RT) for 10 min and then centrifuged at $12,000\times g$ for 10 min at 4 °C. The pellet was washed once with 75 % ethanol and the sample was mixed by vortexing and centrifuged at $7500\times g$ for 5 min at 4 °C. The pellet was briefly dried for 5–10 min. and dissolved in DEPC water (RNase-free water) and quantified. RNA quality as well as quantity were checked by running the samples in a 1.5 % agarose-formaldehyde denaturing gel. Quantification of RNA was done by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Further, all the RNA samples were pooled to purify the mRNA [poly(A)+ RNA] from total RNA and mRNA was purified from 2 mg of total RNA. The purification was performed by using Oligo (dT)-Cellulose beads (Type 7) (Amersham Biosciences).

A cDNA library was constructed from 5 µg of poly(A)+ RNA (isolated from *P. indica* grown in 0.4 M NaCl) in Uni-Zap XR vector using Zap-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's protocol. The resulting phage library contained 1×10^9 plaque-forming units/ml. The insert size of library ranged from 300 to 1500 bp. Using an in vivo excision system, the library was converted to phagemids and transferred in SOLR *E. coli* cells, according to the manufacturer's protocol. Incubation times for mass excision were kept strictly as per the manufacturer's protocol so as not to alter clonal representation.

Plasmids, pBluescript SK-(pBSK) containing cDNA inserts were mass-excised from phage stock of the *P. indica* cDNA library using ExAssist helper phage and propagated in SOLR *E. coli* cells. The cDNAs of *P. indica* were cloned downstream of the *lac* promoter of pBSK plasmids, thus allowing the expression of recombinant proteins upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Over one million *E. coli* recombinant cells from the same bacterial culture were plated on LB agar containing 50 µg ampicillin/ml, 1 mM IPTG and increasing concentrations of salt such as 0.4, 0.6 and 0.8 M NaCl (concentration not permissible for the host bacterial growth). As a control, the cells were also grown in the above medium with no extra salt included. The plates were incubated at 37 °C for 12–16 h.

Bacterial clones which were able to grow on LB plates supplementing with 1 mM IPTG and 0.6 M NaCl at 37 °C (Joshi et al. 2009), were isolated and streaked on the same medium to confirm their abilities to tolerate high concentration of salt. *E. coli* cells with pBSK-vector were used as negative controls. To further confirm the effective contribution of fungal cDNAs to bacterial survival against NaCl and to exclude any association of the observed phenotype with unpredictable chromosomal mutations, the plasmids were purified from these over-expressing colonies (SOLR)

and reintroduced into a different *E. coli* strain (DH5α) (Invitrogen, Life Technologies, Carlsbad, CA, USA) and streaked in LB plates containing IPTG and 0.6 M NaCl. *E. coli* cells transformed with the only vector-pBSK were placed on 0.6 M NaCl plates and was used as a control. Additionally, all the 36 genes transformed in *E. coli* DH5α were also grown with 0.6 M NaCl but without IPTG. Plasmids from these 36 positive colonies were sequenced on both strands by the dideoxy chain termination method, using Sequenase program Version 2.0 (US Biochemicals, Cleveland, OH, USA). The clones of the expression library were found to be in frame with the *LacZα* gene, which is driving their expression in *E. coli*. Sequences were compared to *P. indica* genome data base (Zuccaro et al. 2011) and GenBank database using BLAST N or BLAST X tool (<http://blast.ncbi.nlm.nih.gov/>) to determine the putative functions of the genes.

BLAST X, motif search and gene ontology analysis

The raw sequences were processed by calling the bases from the chromatograms using Finch TV (<http://www.geospiza.com/Products/finchtv.shtml>), and the vector sequences were removed manually. In order to obtain information of functional annotation of identified proteins, the nucleotide sequences of all identified proteins were compared to the NCBI nonredundant (NR) protein database by BLAST X. Sequences with score value more than $e-06$ were categorized into putative functional groups, whereas sequences with score $<e-06$ or with no hits or no significant similarities to the NR peptide database at NCBI, were grouped in the 'nonsignificant matches' category. Minimum values for BLAST e-value and % similarity of the BLAST result were $e-06$ and 55 % respectively and ultimate annotation cut-off value was set to 55. Aside from this, matches to proteins annotated as hypothetical were not counted, as hypothetical genes are often false gene predictions or unknown repetitive elements. Motif search was performed with P-fam database (<http://pfam.sanger.ac.uk>) which is comprised of hand-curated seed protein alignments that are converted to a probabilistic representation using hidden Markov models (HMMs). These HMMs are used to search the protein database for homologues that can be added to the seed to create a comprehensive alignment. In this way, the functional sites and their pattern were determined using P-fam database. GO analysis of cDNA clones was performed with Amigo (<http://amigo.geneontology.org>). Based on the functions identified from the BLAST X results, appropriate GO terms were found using AmiGO, the GO-supported tool for searching and browsing the Gene Ontology database. The results were grouped under three groups: cellular localization, biological process and molecular function.

Quantitative real time-PCR (Q-RT-PCR)

This was carried out to find the relative transcript levels of the putative 36 genes using *P. indica* RNA isolated from fungus grown in KF media and at 400 mM NaCl. For this purpose reverse transcription and subsequent PCR amplification reactions were carried out using power script II reverse transcriptase (Promega Corporation, Madison, WI, USA) and Power SYBR[®] Green PCR Master Mix with ROX reference dye [Takara, Biotechnology (Dalian) Co., Ltd. Dalian, Liaoning province, China] respectively.

Two-step Real time PCR protocol was used where reverse transcription and PCR-mediated cDNA amplification were carried out in subsequent steps in separate tubes. The two-step protocol is preferred when SYBR green is used as a detection dye. The reverse transcriptase reaction was primed with oligo-(dT). PCR reactions were conducted in an ABI 7500 Fast sequence detection system (Applied Biosystems, Life Technologies Carlsbad, CA, USA). A 20 μ l reaction containing the following reagents were used. SYBR[®] premix Ex Taq[™] (Takara) 10 μ l, Primer mix 2.5 μ M (for each pair) 0.8 μ l, ROX reference dye II (Takara) 0.4 μ l, Template, 2.0 μ l, ddH₂O 6.8 μ l. All reaction components were gently mixed in an ABI 96-well Real Time PCR plate and the plate centrifuged at 200 rev/min for 2 min to spin down all reaction components. The Real-Time PCR reactions were carried out with the following thermal profile: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 57 °C annealing for 30 s and 72 °C extension and detection for 30 s. After 40 cycles, the specificity of the amplifications were tested by heating from 60 to 95 °C with a ramp speed of 1.9 °C/min, resulting in melting curves. The reference control genes were measured with three replicates in each PCR run, and their average CT was used for relative expression analyses. Expression data of genes of interest were normalized by subtracting the mean reference gene i.e. translational elongation factor (TEF) CT value from their CT value (Δ CT) (Kumar et al. 2009). The Fold Change values were calculated using the expression, where $\Delta\Delta$ CT represents Δ CT-condition of interest – Δ CT control. The results were converted to a log scale. Primers used for the genes expression analyses are shown in Table 1.

Results

Functional screening of fungus salinity stress-related cDNAs by random over-expression in *E. coli* cells

At 15 days post inoculation (dpi) of *P. indica* in 0, 200, 400 and 600 mM NaCl concentration, the total dry weight increased by 23.4-, 20-, 14- and 10-fold, respectively.

However, at 15 dpi in 800 mM NaCl concentration, only a fourfold increase in dry weight was observed. *P. indica* showed moderate growth up to 600 mM NaCl concentration. Salinity stress negatively affected the total dry weight of *P. indica*. Escalating salt concentration negatively regulated the *P. indica* dry weight by 14.5, 40, 57 and 83 % at 200, 400, 600 and 800 mM NaCl concentration respectively, as compared to 0 mM NaCl at 15 dpi. Significant differences in dry weight were observed with increasing salt stress (Fig. 1). To identify the genes involved in high salt-tolerance, functional screening of salinity-tolerance genes was carried out. For this purpose *E. coli* cells were transformed with phagemids derived from a cDNA library made from RNA isolated from *P. indica* grown at 0.4 M NaCl. A total of over one million recombinant bacterial cells (SOLR *E. coli* cells) were selected on a medium supplemented with NaCl concentrations not permissive for bacterial cell growth. For control, the same number of cells were grown on LB medium (which normally contains 171 mM NaCl) and the result showed a lawn of colonies on the plate. The same number of recombinant bacterial cells plated at 0.4 M NaCl produced ~1600 colonies. We isolated 36 colonies at 0.6 M NaCl and no growth was observed at 0.8 M NaCl. It was noted that the colonies observed at 0.6 M NaCl concentration took 16–20 h to grow and were smaller in size as compared to colonies grown on normal medium.

Salinity tolerance by over-expressing 36 salt-tolerance genes in DH5 α strain of *E. coli*

In order to confirm the role of the 36 genes in conferring salt tolerance to *E. coli*, plasmids were purified from the 36 clones (expressing in SOLR cells) (Table 2) and reintroduced into another strain of *E. coli* DH5 α . Empty pBlue-script vector SK (–) was used as a control. This was done to exclude the possibility of unpredictable chromosomal mutations in SOLR cells, which might have provided salt tolerance. These overexpressing bacterial cells (DH5 α) were induced by IPTG and were subjected to salt (NaCl) stress. In the solid medium all transformants grew on normal LB agar medium containing 171 mM NaCl. All the 36 genes transformant clones grew well at 0.6 M salt stress, while the empty pBluescript vector DH5 α transformants did not grow on 0.6 M NaCl and no growth of all the 36 clones was also observed when grown without IPTG but with the 0.6 M NaCl. Similarly no growth of transformants were observed at 0.8 and 1 M NaCl (data not shown). Additionally, no growth was observed when all 36 colonies were grown for 48 h with different ions like mercury, copper, magnesium, cadmium, potassium chloride of various concentrations (data not shown) which suggest that these 36 colonies are specifically tolerant to NaCl.

Table 1 Primers used for the expression analyses of salt tolerance related 36 genes and translational elongation factor (TEF) gene of *P. indica*

Gene name	Primer name and sequence	Amplicon size (bp)
NAAP-1 amino acid permease	ACPFor 5'-GGTCTCATTCTCGTCTCC-3' ACPRev 5'-CCAACGACATAGCAGGCTTA-3'	152
40S ribosomal protein S20	40SRP FOR 5'-CCGTTTGCCTACCAAGGTTT-3' 40SRP REV 5'-TCGACGTAATCTGCTTGACAATC-3'	100
Cyclopropane-fatty-acyl-phospholipid synthase	SLC9MFor 5'-CGGGATCGTTCACCAAGATT-3' SLC9MRev 5'-ATTGCCATACCGAGCGGATA-3'	150
Polyubiquitin-like protein	PULPFor 5'-CGACGCTTCATCTTGTCT-3' PULPRev 5'-ATCCGGAGGAATACCTTCT-3'	152
Mitochondrial pyruvate dehydrogenase E1	PDEIFor 5'-AGTCGTCGACACCCCATTA-3' PDEIRev 5'-TAGGTTTTTCCGGCCGAGTT-3'	150
Mitochondrial ATP synthase epsilon	ATPSEFor 5'-CAGCAACTGGAGTGCTCACTT-3' ATPSERev 5'-CTTGTCCGTTTTCCACTTTTG-3'	150
Stearoyl-CoA desaturase	D9FADFor 5'-GGTGAGACGCCCTTTGATGA-3' D9FADRev 5'-CTTGGTCGGGTCGACTGGTA-3'	150
ATP:ADP antiporter	AAAPFor 5'-CGCTCAAGCCAGTCGTTCTC-3' AAAPRev 5'-CTACCCACCCCGAGGTCAT-3'	150
NPL4 and ER translocation component	ECPFor 5'-CCGACATCCCAGTCGACTT-3' ECPRev 5'-AATTTCCGGCGAGGAGCTTGT-3'	150
Inosine 5-monophosphate dehydrogenase	I5MDFor 5'-GTCGTTCTCGGTCCAGATGAC-3' I5MDRev 5'-CGGGTCGTGGAACCTGGATAT-3'	150
Translation elongation factor 1-alpha	TEF1For 5'-GGTCAGATTGGCGCTGGTTA-3' TEF1Rev 5'-GGCAGCGTCACCAGACTTG-3'	150
Thiamine pyrophosphate binding domain-containing protein	TPBDPFor 5'-CATCAGCAAAGCGACACAGT-3' TPBDPRev 5'-CCTCCCGTGGCGTCATT-3'	80
40S ribosomal protein S10	18SRPFor 5'-AGGTCATCAAGCCATGCA-3' 18SRPRev 5'-GGGTTGCTGGGACAATCTCA-3'	150
glyceraldehyde 3-phosphate dehydrogenase 27S	27SGDPFor 5'-AAGGGTGCCTCGTATGATGAGA-3' 27SGDPRev 5'-ACCAGCCTTGGCATCAAAGA-3'	150
40S ribosomal protein S12	29SRPFor 5'-GAGGTGATTGGAAGCGTGATC-3' 29SRPRev 5'-TGTGGCACCGATAC-3'	149
BCL-2 associated athanogene 3-like protein	BA3LPFor 5'-AGGGTGCTCTCAAGCCTCAA-3' BA3LPRev 5'-TGGTTCCGTGGTGCTAGTTG-3'	150
Cytochrome P459	CP459For 5'-CGGCTCGCTCTCTCGAGTA-3' CP459Rev 5'-TAGCTCCAAATCTCGTCATGATCT-3'	100
Granulin-like protein	GLPFor 5'-GTCTCTGCCGCTGGGATTT-3' GLPRev 5'-GCCACCATGTGCACATGCAA-3'	150
DNA binding protein	DBPFor 5'-GGCAATTACCCTTCGAGCAA-3' DBPRev 5'-GCCACTGATGTTGGCAAAGA-3'	150
FMN-dependent 2-nitropropane dioxygenase	NPDOFor 5'-CCGCACCGTCATCTTCACT3' NPDORev 5'-ATTTCTGGTCGTTCTCCAAC-3'	150
Pyruvate kinase	PKFor 5'-CGTTCGATCATCACCGTTA-3' PKRev 5'-AGGCCAAAGCGGATTCTGTT-3'	150
UBI4—ubiquitin	PUPFor 5'-CTACTGGCAAGACGATCACTCT-3' PUPRev 5'-GCGTCGACTCCTTTTGAATATTATAGT-3'	178
Unknown	UNKFor 5'-AGCTGCGACAATGCCAGACT-3' UNKRev 5'-TGAAACAATATAACCTCGCAGGAA-3'	150
60S ribosomal protein	60SRPFor 5'-TGTCCGCAAGCTGATCAAAG-3' 60SRPRev 5'-GGCATAACGACCTCTGCAGTA-3'	150
Myosin regulatory light chain cdc4	EFHPFor 5'-CCAGAGGAGTTTATTCGAGGATTC-3' EFHPRev 5'-GCCAATCTGAACACCCTTGAG-3'	150
Expansin family protein	EXFPFor 5'-TTTTTGACACACGGTGAT-3' EXFPRev 5'-GGCAACACGGAGTGGGATTA-3'	150

Table 1 continued

Gene name	Primer name and sequence	Amplicon size (bp)
Metallothionein	METTFor 5'-ATGATCTCTGAAACCATTGTCCCG-3' METTRev 5'-TTA GCA CTT GCA CTC GCC CGG-3'	102
31S hypothetical protein	31SHPFor 5'-ACGACTGTCCGTCACAATCG-3' 31SHPRev 5'-CTGGTCCTTCAAGCGGATGT-3'	150
Chitinase-like protein	CLPFor 5'-CGAAACAACGGCGGAGATTA-3' CLPRev 5'-CACAAGCTCCCCCTTGAGTCT-3'	150
33S hypothetical protein	33SHPFor 5'-CCTACGTCGTCCTCGATTG-3' 33SHPRev 5'-TTGCTTCCATTACAGACATGA-3'	150
35S hypothetical protein	35SHPFor 5'-CAAAGCACCTTCTCTGGTGT-3' 35SHPRev 5'-CGAAGGCCAGCTGCAT-3'	166
Cytochrome P450-like	CP450For 5'-TCACTCGGCCATGATCAG-3' CP450Rev 5'-ATGTGGGCTGGTTGGGAAA-3'	150
NADH dehydrogenase ubiquinone Fe-S 7 protein-like protein	NDUFor 5'-GCATATGGCTGCTGCTCGTT-3' NDURev 5'-GGTTCGGGCATCTGATCGTA-3'	150
48S hypothetical protein	48SHPFor 5'-ACACCTACAACGGGATCTGCAT-3' 48SHPRev 5'-AACAAACGGCGACGCTTGA-3'	150
24S cyclophilin	24SCYFor 5'-CCATCGCATATTCTCAGTT-3' 24SCYRev 5'-GCATTGGCCATGGAGAGAAG-3'	150
Unknown	UNKNFor 5'-CCAGTCTGCCACGGATGAGT-3' UNKNRev 5'-GACGATGGATTGTCGGGATT-3'	150
Translation elongation factor (tef) control	PitefFOR 5'-TCGTCGCTGCAACAAGATG-3' PitefREV 5'-GAGGGCTCGAGCATGTTGT-3'	150

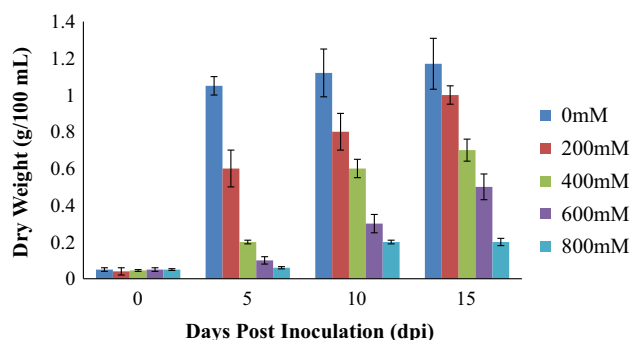


Fig. 1 Growth of *P. indica* at different salt concentrations. Fungus was grown at different salt (NaCl) concentrations (0, 200, 400, 600 and 800 mM provided in AMM) at 150 rev/min and 30 ± 2 °C. Growth of *P. indica* was measured in terms of dry weight/100 ml by collecting fungal mycelium at 0, 5, 10, 15 and 20 days. *Note:* values are mean \pm SD of three replicates ($n = 3$) and experiment was repeated twice

Plasmids were rescued from the 36 colonies grown under 0.6 M NaCl and the insert were sequenced.

BLAST X, motif search and GO annotation

Sequences of the inserts ranging from 300 to 1500 bp were compared with data from the *P. indica* genome and further analysed by BLAST N and BLAST X to identify the putative functions of the corresponding genes. After

screening, several anti-stress genes were successfully isolated, such as those encoding cyclophilin, ribosomal proteins, EF hand protein, DNA-binding proteins, desaturase, sphingolipid methyltransferase etc. The DNA sequences were submitted to the GenBank database under accession numbers FJ440106, FJ440107, FJ668531, FJ668532, FJ668533, FJ668534, FJ695614, FJ695615, FJ695616, FJ695617, FJ695618, FJ695619, GQ214002, GQ214003, GQ214004, GQ214005, FJ712689, FJ716809, FJ716810, FJ746637, GQ154470, GQ154471, GQ129457, GQ129458, FJ944819, FJ944820, FJ944821, GQ257365, GQ257366, GQ257367, GQ257368, GQ257369, GQ257370, GQ257371, GQ257372. Pfam results were analysed to have a correlation between the BLAST results and the domain found in the gene. For the identification of motifs, cut-off was set to be $1e-04$ and motifs with a score less than this were treated as non-significant. To obtain as much functional annotation information as possible, the identified cDNA clones were compared to those in the GO database by using online tool AmiGO. From the initial BLAST X analyses for good hits, 29 of 36 cDNA clones were annotated with specific GO terms (Supplementary material Table 1). In total, 20 clones were considered to be significant matches to characterized GO proteins, with an E-value $<1e-04$. As shown in Fig. 2a–c, the putative annotations were grouped into three ontologies viz., cellular component, biological process and

Table 2 List of high salinity stress tolerant genes of *P. indica* obtained after random overexpression in *E. coli* DH5 α

S. no.	Gene name (accession no.)	Location	Biological process	Molecular function	Motif
<i>Complete genes</i>					
1.	Cyclophilin (GQ214003)	Cytosol, nucleus	Protein folding	Unknown	Cyclophilin type peptidyl-prolyl <i>cis-trans</i> isomerase
2.	Stearoyl-CoA desaturase (FJ695614)	Intrinsic to ER membrane	Unsaturated fatty acid biosynthetic process	Stearoyl-CoA 9-desaturase activity	Cytochrome b5-like heme/steroid binding domain
3.	Myosin regulatory light chain cde4 (FJ944820)	Ribosomal, intracellular	Translation	Structural constituent of ribosome, calcium ion binding	EF hand
4.	Eukaryotic ATP:ADP antiporter (FJ695615)	Mitochondrial inner membrane, mitochondria	Mitochondrial transport	ATP:ADP antiporter activity	Mitochondrial carrier protein domains
5.	Inosine 5-monophosphate dehydrogenase (FJ695617)	Cytoplasm	Biosynthesis of guanosine nucleotide	IMP dehydrogenase activity	IMP dehydrogenase/GMP reductase domain
6.	Mitochondrial ATP synthase epsilon chain domain-containing protein (FJ668534)	Mitochondria	Mitochondrial ATP synthesis coupled proton transport	Hydrogen ion transporting ATP synthase activity, rotational mechanism, proton transporting ATPase activity	ATP-synt Eps family
7.	Mitochondrial pyruvate dehydrogenase E1 component (FJ668533)	Mitochondria	Acetyl-CoA biosynthetic process from pyruvate	Pyruvate dehydrogenase (acetyltransfering) activity	Transketolase pyrimidine binding domain, Transketolase, C-terminal domain
8.	NADH dehydrogenase ubiquinone Fe-S 7 protein-like protein (GQ257371)	Inner mitochondrial membrane	Electron transport system	Transfer electrons from NADH to co-enzyme Q	8 Fe-S cluster, hydrophilic peripheral redox centres domain
9.	40S ribosomal protein s20 mRNA (FJ440107)	Cytosol	Translation	Structural constituent of ribosome	Ribosomal protein S10p/S20
10.	40S ribosomal protein s10 (GQ214002)	Cytosol	Translation	Structural constituent of ribosome	Plectin/S10 domain
11.	60S ribosomal protein L19 (FJ944819)	Cytosol	Translation	Structural constituent of ribosome	Ribosomal protein L19e
12.	Cyclopropane-fatty-acyl-phospholipid synthase (FJ668532)	Mitochondrial inner membrane	Ubiquinone biosynthetic process	Hexaprenyldi-hydroxybenzoate methyltransferase activity	Cyclopropane-fatty-acyl-phospholipid synthase motifs
13.	Polyubiquitin-like protein (FJ668531)	Cytoplasm	Protein ubiquitination (mono, di, poly), cellular response to heat, response to stress	ATP-dependent protein binding, protein tag	Ubiquitin
14.	Cytochrome P459 (FJ716809)	–	–	–	–
15.	Granulin-like protein (FJ716810)	Intact to plasma membrane	Cell growth	Secreted and glycosylated peptide	Granulin motif
16.	Metallothionein (GQ257365)	Golgi apparatus membrane	Primary metal storage, transport and detoxification	Metal chelating peptide	Cysteine-rich
17.	3 IS hypothetical protein (GQ257366)	–	–	–	–

Table 2 continued

S. no.	Gene name (accession no.)	Location	Biological process	Molecular function	Motif
18.	Chitinase-like protein (GQ257367)	Plasma membrane, vacuole	Break down of glycosidic bonds in chitin, carbohydrate metabolism	Glycosidase, hydrolase	Chitin binding and chitinase activity
19.	35S hypothetical protein (GQ257369)	–	–	–	–
20.	Cytochrome P450 (GQ257370)	Endoplasmic reticulum, cell organelles	Oxidation–reduction process	Electron carrier activity, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	Iron ion binding domain, heme binding domain
21.	48S hypothetical protein (GQ257372)	–	–	–	–
22.	Unknown protein (GQ925366)	–	–	–	–
<i>Partial genes</i>					
23.	NAAP-1 amino acid permease NAAP1 (FJ440106)	Plasma membrane	Amino acid transport, oxidation reduction, transmembrane transport, phosphorus metabolic process	Amino acid transmembrane transporter activity	Amino acid permease motif
24.	DNA-binding protein (FJ746637)	Cytoplasm	Unknown	Unknown	Domain of unknown function
25.	NPL4—nuclear protein localization factor and ER translocation component (FJ6956116)	Nuclear envelope, endoplasmic reticulum network	Cell death, ER associated protein catabolic process	No function known i.e. no evidence	NPL4 family
26.	Glyceraldehyde 3-phosphate dehydrogenase (GQ214004)	Cytosol, fungal-type cell wall	gluconeogenesis, glycolysis, stress-activated protein kinase signaling pathway	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity, protein binding	Glyceraldehyde 3-phosphate dehydrogenase, C-terminal domain
27.	Pyruvate kinase (GQ154471)	Cytosol, mitochondrion	Pyruvate metabolic process	Pyruvate kinase activity	Pyruvate kinase, barrel domain family, alpha/beta domain
28.	UBI4—ubiquitin (GQ129457)	Cytoplasm	cellular response to heat, protein deubiquitination, mono, polyubiquitination, response to stress	ATP-dependent protein binding, protein tag	Ubiquitin family motifs
29.	Ribosomal protein 40S S12 (GQ214005)	Nucleolus, nucleus, cytosol	Translation, ribosomal small subunit export from nucleus	Structural constituent of ribosome	Ribosomal protein S19
30.	Translation elongation factor 1-alpha (FJ695618)	Cytosol	Translational elongation, tRNA export from nucleus	GTP binding, GDP binding, Translation elongation factor activity	Elongation factor Tu C-terminal domain

Table 2 continued

S. no.	Gene name (accession no.)	Location	Biological process	Molecular function	Motif
31.	BCL-2 associated athanogene 3-like protein (FJ712689)	Cytoplasm mitochondria	Modulate heat shock proteins 70 (Hsc70/Hsp70) and Bcl-2 functions, either positively or negatively	Interact with ATPase binding domain of heat shock proteins 70 (Hsc70/Hsp70) and Bcl-2;	Hsp70/Hsc70 interaction domain/ binding domain
32.	Thiamine pyrophosphate binding domain-containing protein (FJ695619)	Membrane, cytoplasm	Catalytic activity of many enzymes	Acts as a coenzyme of several carboxylases and de-carboxylases, trans ketolases, and alpha-oxoacid dehydrogenases	Interacts selectively and non-covalently with thiamine pyrophosphate
33.	FMN-dependent 2-nitropropane dioxygenase (GQ154470)	Cytoplasm	Nitrogen metabolism, Oxidation–reduction process, nitronate monooxygenase activity	Catalyses the oxidation of nitroalkanes into their corresponding carbonyl compounds and nitrite using either FAD or FMN as a cofactor	The family of oxidoreductases
34.	Unknown protein (GQ129458)	–	–	–	–
35.	Expansin family protein (FJ944821)	Extra-cellular	Cell wall stress relaxation and its irreversible extension during growth	Binds to plant cell walls and induces plant cell wall extension during plant colonization	Family of closely related nonenzymatic proteins found in the cell wall
36.	33S hypothetical protein (GQ257368)	–	–	–	–

All the 36 genes sequences have been deposited in the NCBI (Acc. number are shown in bracket). Identification of the genes and the function, location analysis was done with the *P. indica* genome and NCBI data base

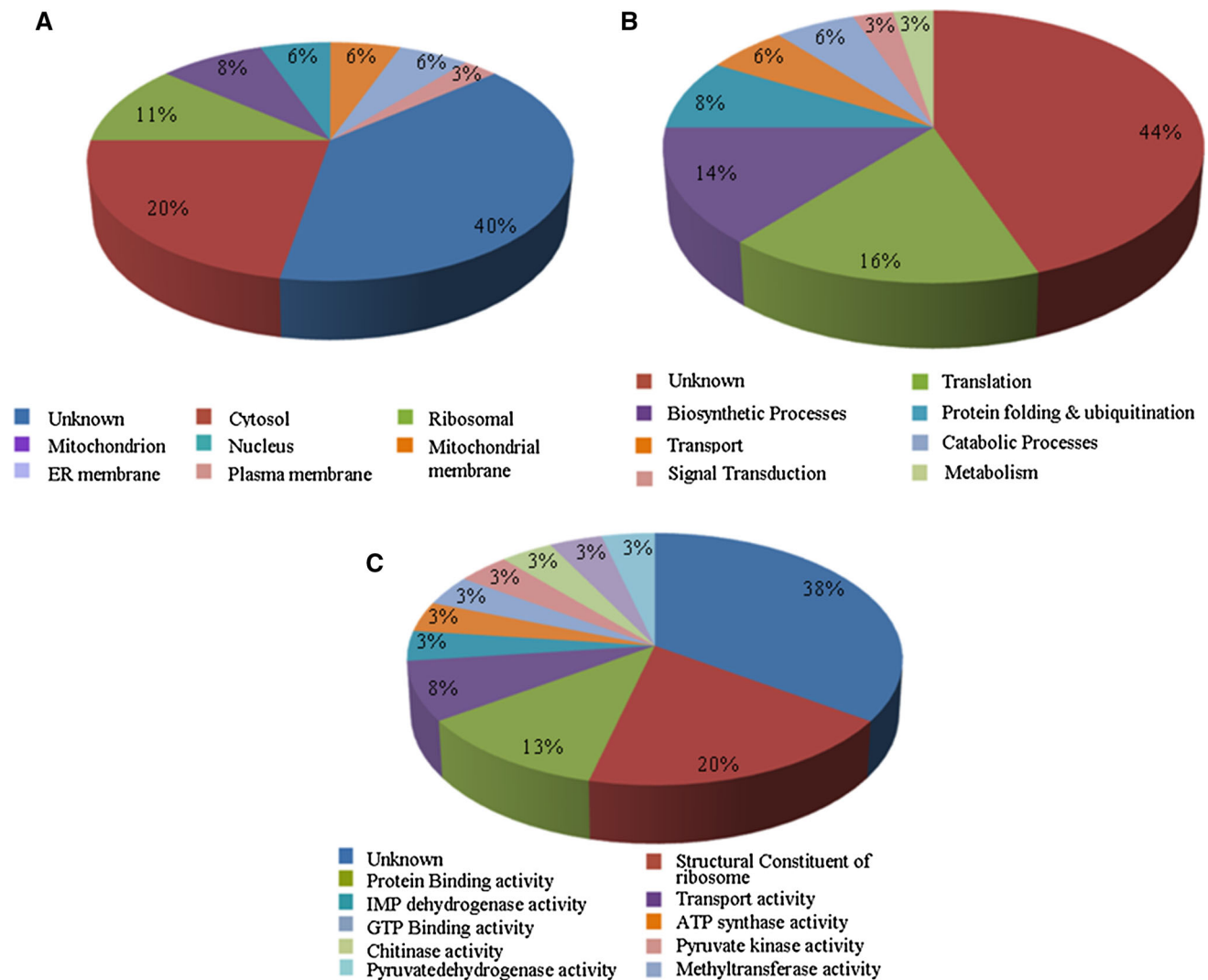


Fig. 2 Distribution of **a** cellular component, **b** biological process, **c** molecular function assignment for *P. indica* cDNA by GO annotation. For this GO annotation of *P. indica* cDNA was generated by using Amigo online software analysis tool and *Saccharomyces*

cerevisiae as GO template. A total 36 sequences were annotated. Next to the each functional category is the colour code which signifies the respective process, component and function

molecular function. Among those sequences that could be assigned a functional classification, the largest categories were ribosome and protein regulation.

Expression analyses of salt-tolerance-conferring genes

To analyse the expression of the selected 36 genes in *P. indica*, Q-RT-PCR was performed (Fig. 3). Induction by salt treatment was found for 6 genes encoding cyclophilin, stearoyl-CoA desaturase, thiamine pyrophosphate-binding domain-containing protein, BCL-2 associated athanogene 3-like protein, cytochrome P-450 and 60S ribosomal protein genes up-regulated in 400 mM NaCl-treated *P. indica* as compared to the *P. indica* grown in only KF media

(Fig. 2). We have found cyclophilins and stearoyl-CoA desaturase genes 1.5- and 3-fold up-regulated respectively at high salt treatment as compared to *P. indica* grown without salt treatment. The expression pattern of all the 36 genes is shown in Table 3.

Discussion

Piriformospora indica is a root-colonizing fungus which is able to increase the biomass and yield of crop plants and to induce local and systemic resistance to fungal diseases and tolerance to abiotic stress (Waller et al. 2005; Kumar et al. 2009). Fungal stress-signalling pathways have evolved rapidly in a niche-specific fashion that is independent of

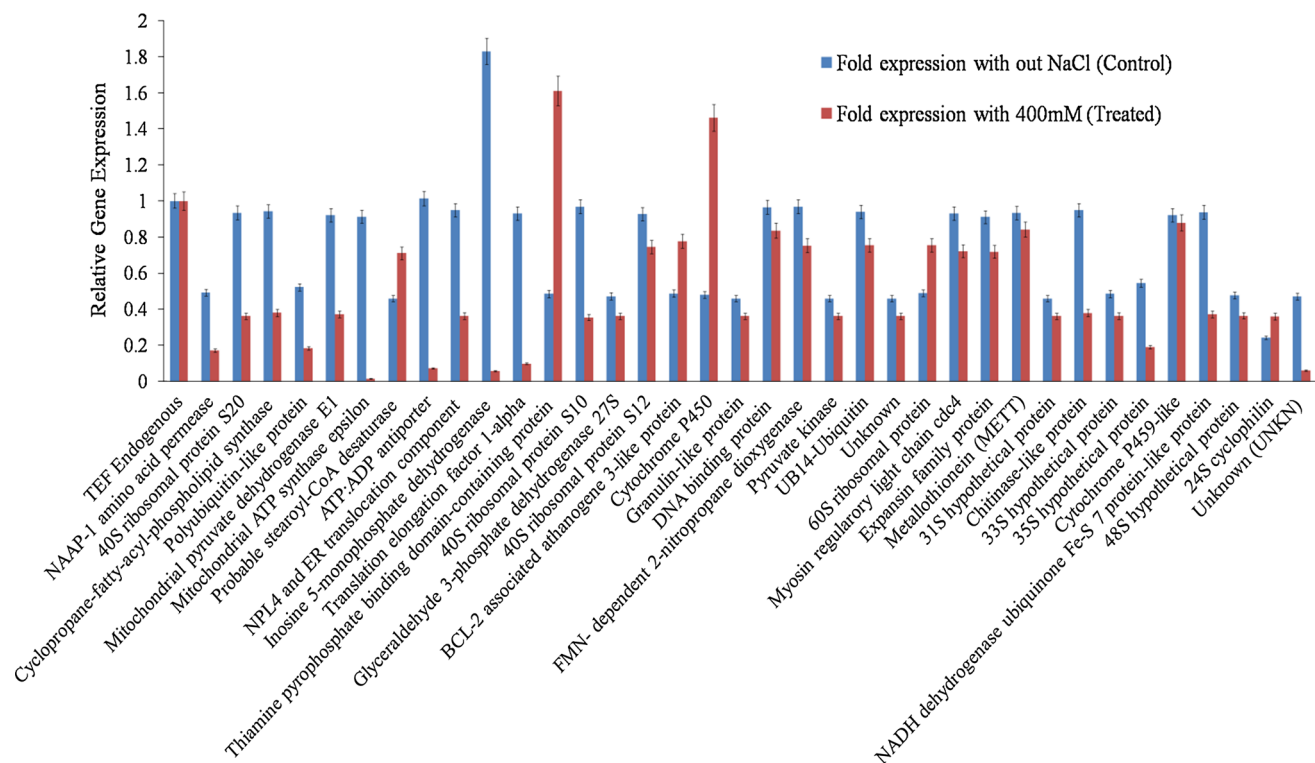


Fig. 3 Q-RT-PCR. Expression analyses of 36 genes using RNA from *P. indica* grown with 400 mM NaCl or without NaCl. RNA was extracted from *P. indica* and subjected to Q-RT-PCR using specific primers and SYBR green I. The comparative Ct method was applied

phylogeny and are relatively well conserved (Nikolaou et al. 2009). Hence, the stress response mechanisms reported in the case of other fungi might also apply to *P. indica*, and the genes responsible might also play a similar role. An overall knowledge of the bioinformatics, biochemistry and molecular biology of the fungus is therefore necessary to understand the mode of action of how the endophyte provides salt-stress tolerance. The principle of the screening method was based on the acquisition of NaCl salt-stress tolerance of the *E. coli* cells carrying (or expressing) the fungal genes. We have isolated 36 clones and the DNA sequences of these clones were analysed.

Out of 36 clones, 29 clones were found to be hits with non-significant BLAST X results, which may be due to the high cut-off value, which was set to increase the level of significance of matches with sequences in the databases, whereas few genes were unknown proteins that might be attributed to the possibility that many unique salinity-tolerance genes have not been annotated in the databases. These proteins may represent new salinity-tolerance gene candidates from *P. indica*. Research on their functions may shed light on new mechanisms of salinity tolerance in this fungus. Comparison with the GO database was performed to get more information on the cellular components and the biological processes of these clones. Annotation information

to analyse the data. Fold expression compared to that of the Translational Elongation Factor (TEF) gene in KF medium; fold expression compared to the TEF gene in KF medium with 400 mM NaCl added

was obtained for 64 % of the identified proteins through GO database comparison and it was clear that the identified clones were predicted to cover all main organelles such as nucleus, mitochondrion, plasma membrane, and endoplasmic reticulum. Proteins were sorted into main functional classes based on GO data. As shown in Fig. 2a–c, the putative annotations were grouped into three ontologies: cellular component, biological process, and molecular function. Among those sequences that could be assigned a functional classification, the largest categories were ribosomes and protein regulation. Failure in GO term assignment was due to a negative result in the BLAST search, the absence of GO annotation in any of the BLAST hits or because the short length of certain partial sequences. The sequences that have putative start codons and hence have coding potential but do not share significant homology to deposited sequences could represent conserved genes that are not yet described in other fungi or genes that are unique to *P. indica*.

Further, Q-RT-PCR was done to check the expression of all the 36 genes using *P. indica* RNA (Fig. 3). Out of 36 genes only 6 genes were found up-regulated in salt-treated *P. indica* as compared to the *P. indica* grown with no salt treatment. Cyclophilins, which are cytosolic and constitute a family of proteins involved in many essential cellular functions, were found to be up-regulated. Cyclophilins are

Table 3 Expression pattern of *P. indica* genes in response to salt stress

Name of genes	Fold up-regulation as compared to control	Fold down-regulation as compared to control
Probable stearyl-CoA desaturase	1.5	
Thiamine pyrophosphate binding domain-containing protein	3.3	
BCL-2 associated athanogene 3-like protein	1.5	
Cytochrome P450	3.0	
60S ribosomal protein	1.5	
24S cyclophilin	1.4	
APC permease-like protein		0.34
40S ribosomal protein		0.38
Sphingolipid C9-methyltransferase		0.40
Polyubiquitin-like protein		0.34
Mitochondrial pyruvate dehydrogenase E1		0.40
Mitochondrial ATP synthase epsilon		0.01
ATP:ADP antiporter		0.06
ER-associated catabolism-related protein		0.38
Inosine 5-monophosphate dehydrogenase		0.030
Translation elongation factor 1-alpha		0.10
18S 40S ribosomal protein		0.36
Glyceraldehyde 3-phosphate dehydrogenase 27S		0.76
29S 40S ribosomal protein		0.80
Granulin-like protein		0.78
DNA binding protein		0.86
2-nitropropane dioxygenase		0.77
Pyruvate kinase		0.78
Polyubiquitin protein		0.80
Unknown		0.78
EF-hand protein		0.77
Expansin family protein		0.78
Metallothionein (METT)		0.90
31S hypothetical protein		0.78
Chitinase-like protein		0.39
33S hypothetical protein		0.74
35S hypothetical protein		0.34
Cytochrome P450-like		0.95
NADH dehydrogenase ubiquinone Fe-S 7 protein-like protein		0.39
48S hypothetical protein		0.76
Unknown (UNKN)		0.12

a conserved family of proteins present in bacteria, fungi, plants, and animals and are best known for being the cellular target of the immunosuppressive drug cyclosporin A. The role of cyclophilins has been reported in many cellular processes, such as response to environmental stresses, cell cycle control, regulation of calcium signalling, control of transcriptional repression and fungal growth during the infection in plants (Matouschek et al. 1995; Joseph et al. 1999; Viaud et al. 2002). In the case of *Aspergillus nidulans* a role of cyclophilin B has been proposed during

growth in high-stress environments (Joseph et al. 1999). In the case of *Magnaporthe grisea* CYP1, cyclophilin plays an important role in the development of asexual reproductive structures and cellular turgor generation in appressoria (Viaud et al. 2002). In addition, two cyclophilin A-encoding genes were identified from the human pathogenic fungus *C. neoformans* (Wang et al. 2001) and shown to play roles in cell growth, mating, and virulence. However the role of cyclophilins has not been reported in the case of *P. indica*. As we have found this gene up-regulated,

we speculate that it might help *P. indica* during salt-stress conditions. We have also found thiamine pyrophosphate (TPP)-binding domain-containing protein up-regulated under salt stress. TPP-binding domain-containing proteins have been reported to be involved in metabolic processes and catalytic activity of many enzymes related to energy metabolism. The protein interacts selectively and non-covalently with thiamine pyrophosphate, and acts as a coenzyme of several carboxylases and decarboxylases, transketolases, and alpha-oxoacid dehydrogenases. These enzymes are related to energy metabolism, pentose phosphate pathway, glycolysis and mitochondria (Maas and Bisswanger 1990). In the case of *P. indica* we suggest that this gene may be involved in energy metabolism during the salt-stress condition.

The other salt-up-regulated gene encodes a Bcl-2-associated athanogene (BAG)-like protein, an evolutionarily conserved protein family, found to interact with the ATPase binding domain of heat shock proteins 70 (Hsc70/Hsp70) and to modulate their functions, either positively or negatively (Pascale et al. 2010). The BAG domain is an evolutionarily conserved region located at the C terminus of the BAG-family proteins and has been described and/or proven in a variety of organisms. Human BAG-1 was the first member of this family discovered through a screen for Bcl-2 binding proteins (Takayama et al. 1995). All BAG proteins share a common Hsp70/Hsc70 interaction domain/binding domain (BD), but generally differ in the N terminal region, which imparts specificity to particular proteins and pathways. The ubiquitin-like domain at the N terminus of human BAG proteins (BAG1 and BAG6) is probably functionally relevant and conserved in yeast, plants, and worms (Doukhanina et al. 2006). The role of BAG proteins was suggested to be involved in the regulation of diverse physiological processes including apoptosis, stress responses, and the cell cycle. We suggest that in the case of *P. indica*, BAG may also contribute towards these functions. However it needs detailed study.

Cytochrome P-450 is also found to be up-regulated in response to salt-treated *P. indica*. This superfamily is a large and diverse group of enzymes found in all kingdoms of life that have monooxygenase and oxidoreductase activity, electron carrier activity and acting on paired donors, with incorporation or reduction of molecular oxygen. The substrates of cytochrome P-450 enzymes include metabolic intermediates such as lipids and fatty acids. In plants, these proteins are important for the biosynthesis of several compounds such as hormones, defensive compounds and fatty acids. The role of cytochrome P-450 was predicted to be related to dissimilatory nitrite reduction by *Fusarium oxysporum* (Kizawa et al. 1991). Up-regulation of this gene during salt stress in *P. indica* may help in energy metabolism, membrane repair and fatty acid synthesis.

We have also found stearoyl-CoA desaturase (also known as delta-9-fatty acid desaturase or FAD) gene to be up-regulated. Stearoyl-CoA desaturase has been reported to be involved in the biosynthesis of unsaturated fatty acids and helps in maintaining the fluidity of cell membrane in case of thermal stress (Nakashima et al. 1996). We speculate that Stearoyl-CoA desaturase might help *P. indica* in maintaining the fluidity of cell membrane under salt stress and thereby preventing the cell membrane from rupturing that might occur as a result of excessive amounts of Na⁺ ions. The sixth gene is a 60S ribosomal protein which plays an important role in the elongation step of protein synthesis in the ribosome and up-regulation of 60S ribosomal protein may help in stabilization of ribosome assembly and protein synthesis (Nomura 1999).

In addition to the up-regulation of six genes, we found that genes mainly related to metabolism (n = 10), repair (n = 3), cytoskeleton (n = 3), were found to be down-regulated. The majority of genes are involved in metabolism and maintenance, which require energy. We hypothesize that these genes found to be down-regulated may be involved in energy production and that during these processes now can be utilized by the fungus for other cellular functions which will be helpful in order to develop resistance during high salt stress. Therefore down-regulation of these genes is required during high salt tolerance. However, their roles need further validation.

These 36 genes represent new candidates for salinity stress in *P. indica* which might be necessarily involved in a specific mechanism of salt tolerance. It is possible that these genes are not sufficient for the overall stress-tolerance phenomenon. It remains to be tested for in vivo functions for further clarification of their role. Yet, this study will enrich our understanding of salt tolerance mechanism in *P. indica*, and this understanding will improve the application of the endophyte *P. indica* for crop improvement in salinity environment. Though we have found the up-regulation of six genes during high salt stress, however, a detail study is required to know their role during the interaction of *P. indica* with the host plant. Current data gives us the idea that analogous salinity-stress tolerance genes exist in both unicellular organisms and plants, suggesting that common mechanisms for salinity tolerance are emerging across the phylogenetic spectrum. Therefore, isolating novel salinity stress-induced genes from fungi and checking their salinity tolerance in bacteria could be a faster approach for the functional validation of genes in salt tolerance.

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Conflict of interest We have no conflict of interest.

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