

Genome-Wide Characterization of Peptidyl-Prolyl Cis-Trans Isomerases in Penicillium and Their Regulation By Salt Stress in a Halotolerant P. Oxalicum

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Abstract

Peptidyl-prolyl *cis-trans* isomerases (PPlases) are the only class of enzymes capable of *cis-trans* isomerization of the prolyl peptide bond. The PPlases, comprising of different families viz., cyclophilins, FK506-binding proteins (FKBPs), parvulins and protein phosphatase 2A phosphatase activators (PTPAs), play essential roles in different cellular processes. Though PPlase gene families have been characterized in different organisms, information regarding these proteins lacks in *Penicillium* species, which are commercially an important fungi group. In this study, we carried out genome-wide analysis of PPlases in different *Penicillium* spp. and investigated their regulation by salt stress in a halotolerant strain of *Penicillium oxalicum*. These analyses revealed that the number of genes encoding cyclophilins, FKBPs, parvulins and PTPAs in *Penicillium* spp. varies between 7-11, 2-5, 1-2, and 1-2, respectively. The halotolerant *P. oxalicum* depicted significant enhancement in the mycelial PPlase activity in the presence of 15% NaCl, thus, highlighting the role of these enzymes in salt stress adaptation. The PPlase activity in *P. oxalicum* was associated with the expression of *PoxCYP18*, *PoxCYP23*, *PoxCYP41*, *PoxFKBP12-2*, and *PoxFKBP52* genes. Characterization of PPlases in *Penicillium* spp. will provide an important database for understanding their cellular functions and might facilitate their applications in industrial processes through biotechnological interventions.

Introduction

The peptide bonds not preceding proline are almost always *trans* in folded proteins, but about 10-15% of all Xaa-Pro (Xaa: other bulky amino groups preceding proline) peptide bonds show *cis* conformation^{1,2}. Conversion of Xaa-Pro bond from *cis* to *trans* conformation, imperative for the correct folding of proteins, is a slow rate-limiting step and requires intervention of PPlases³. The PPlases are categorized into different classes viz., cyclophilins, FKBPs and PTPAs. While cyclophilins bind cyclosporin A (CsA), the FKBPs show affinity for FK506 and rapamycin. The PPlase activity of parvulins is sensitive only to juglone and is not affected by either CsA or FK506⁴. The PPlases that contain both cyclophilin and FKBP domains have also been reported⁵. The PTPAs also exhibit PPlase activity, but are structurally and biochemically distinct from cyclophilins, FKBPs and parvulins and, hence categorized as a different class⁶.

The cyclophilins, defined by a conserved cyclophilin-like domain (CLD), are ubiquitously observed in bacteria to higher organisms⁷, and are encoded by large gene families, with the number ranging from eight in *Saccharomyces cerevisiae* to 19 in humans, 89 in wheat and 91 in *Brassica napus*⁸⁻¹². The FKBPs also constitute a multigene family. Compared to four in *S. cerevisiae*, up to 18 and 29 different family members have been reported in humans and rice, respectively¹³⁻¹⁵. A characteristic feature of FKBPs is the presence of an approximately 110 amino acid (AA) long FK506-binding domain (FKBD) that acts as a receptor of FK506 and rapamycin. The repertoires of parvulins and PTPAs are limited, with only one and three parvulins reported in *Escherichia coli* and humans, respectively^{4,16-18}. Likewise, only one PTPA gene was observed in humans, compared to two in *S. cerevisiae*^{6,19}.

Besides being implicated in several essential cellular processes, such as receptor complex stabilization, plant growth and development, RNA processing, etc., several cyclophilins have also been implicated in abiotic stress adaptation^{7,20-26}. Role in abiotic stress response has also been demonstrated for FKBPs such as wFKBP77 and VfFKBP15 in wheat and *Vicia faba*, respectively, and *Scf.FKBP12* in *Scenedesmus* sp.²⁷⁻²⁹. These observations imply that PPlase genes may serve as suitable candidates for enhancing the abiotic stress tolerance of microbes and plants for industrial processes and agricultural applications and, thus, warrant further investigations.

Eco-friendly solutions for industrial production of different biomolecules entail the application of microbial cells. To this purpose, *Penicillium* has long been used for the production of specialized cheese, antibiotics, enzymes and a wide range of other biologically active metabolites³⁰⁻³². However, exposure to NaCl, an integral component of the media used for growth during various fermentation processes, often results in salt stress and adversely affects the growth and metabolism of microbial cells due to Na²⁺ toxicity and osmotic stress. Further, the sensitivity to salt stress also limits the use of seawater as a sustainable substitute for freshwater in industrial processes³³. Therefore, to develop viable bioprocesses under high salt conditions, it is imperative that the role of PPlases in salt stress tolerance be investigated in the microbial strains.

Halotolerance in fungi is attributed to several different mechanisms viz., maintenance of plasma membrane fluidity and Na⁺ homeostasis, accumulation of compatible osmolytes, and expression of genes implicated in mitochondrial biogenesis and metabolism³⁴⁻³⁷. Synthesis of chaperones such as heat shock proteins and PPlases also provides protection against stress-induced damage to the cell^{7,25,26,38,39}. Though PPlases have been characterized in several fungi⁴⁰, and implicated in stress response in *Aspergillus* and *Geobacillus*⁴¹⁻⁴³, information about these proteins is lacking in *Penicillium*. Therefore, in the present study, we carried out *in silico* characterization of cyclophilin, FKBP, parvulin and PTPA gene families in *Penicillium* spp. and analysed the effect of salt stress on intracellular PPlase activity and expression of these genes in a halotolerant strain of *P. oxalicum* that is able to grow in the presence of up to 15% salt (NaCl). These findings revealed that the number of cyclophilins, FKBPs, parvulins, and PTPA in different *Penicillium* species vary between 7-11, 2-5, 1-2 and 1-2, respectively. The halotolerant isolate of *P. oxalicum* exhibited significant enhancement in the mycelial PPlase activity under salt stress which was also accompanied by a substantial increase in the expression of a cyclophilin gene, *PoxCYP18*. These studies are the first to identify and characterize different PPlase gene families in *Penicillium* and their possible role in salt stress response. The results of these investigations will provide an important database for further elucidation of the role of PPlases in different aspects of growth and development in *Penicillium* which may lead to their potential exploitation for different commercial processes.

Materials And Methods

The halotolerant fungal strain HP1, used in this study, was isolated from the leaves of healthy plants of *Citrus limon* and identified by the Microbial Type Culture Collection (MTCC) Chandigarh, India. The culture was identified using microscopic and molecular methods. The slide culturing technique was performed to determine the microscopic characters of the fungus and its morphological characterization according to the standard taxonomic key characters. Identification of the culture on the molecular basis was carried out by amplification of the internal transcribed spacer (ITS) region containing 5.8S rRNA that

yielded an amplicon of 338 bp. Sequencing of the amplicon and its subsequent analysis by basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) server revealed its maximum similarity with *P. oxalicum* (NR_121232.1) (Supplementary Fig. S11).

The mycelial production was carried out by inoculating one plug (8 mm diameter) of actively growing fungal culture in 250 ml Erlenmeyer flasks containing 50 ml Sabouraud production medium that either lacked or contained NaCl (15%). The inoculated flasks were incubated at 30°C for 10 days on a rotary shaker at 180 rpm and withdrawn at different time intervals for different analyses. For dry weight estimations, the cultures (50 ml) were filtered through Millipore membrane filters (0.45 mm) under vacuum and dried at 80°C until constant weight. The growth curves were constructed from the results obtained from two independent experiments, each carried out in triplicate, and were used to determine the final fungal biomass yield. The effect of salt on colony diameter was analyzed by inoculating Sabouraud agar plates containing 15% NaCl with fungal culture, followed by incubation at 30°C. The fungal growth was monitored daily for ten days by measuring the diameters of individual colonies.

Estimation of peptidyl-prolyl *cis-trans* isomerase (PPIase) activity

The total soluble proteins were extracted from the filtered mycelia of *P. oxalicum* by adding lysis buffer [50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA (pH 8), 10% Glycerol, 1 mM phenylmethylsulfonyl fluoride] followed by sonication with 10 s on and 5 s off for 5 min at 4°C. The homogenate was centrifuged at 6500 rpm for 30 min at 4°C and the supernatant was collected and stored at -20°C after filtering through 0.45 µm filters. The total soluble proteins were estimated according to Bradford's method⁸⁰ using bovine serum albumin as the standard. The PPIase activity in the crude extracts was estimated by a chymotrypsin-based coupled reaction at 15°C for 360 s⁸¹. The 1 ml assay mixture contained 80 µM succinyl-ala-ala-pro-phe-p-nitroaniline as test peptide, assay buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 0.05% Triton X-100] and different concentrations of the crude protein. The reaction was initiated by addition of chymotrypsin at a final concentration of 300 µg/ml. The absorbance change at 390 nm was monitored at 15 °C by using Spectrophotometer (Perkin-Elmer Lambda Bio 25) equipped with Peltier temperature control system. The cyclophilin- and FKBP-associated PPIase activities were determined by the extent of inhibition of reaction in the presence of the specific inhibitors CsA and FK506, respectively. The inhibitors were added to the assay mix 30 min before starting the reaction and incubated at 4°C. The PPIase activity was calculated as the product of the difference in the catalysed and uncatalysed first-order rate constants (derived from the kinetics of the absorbance change at 390 nm) and the amount of substrate in each reaction.

Bioinformatics analysis

The amino acid sequences of cyclophilins, FKBP, parvulins and PTPAs of 24 different *Penicillium* spp. were retrieved from the NCBI server (<http://www.ncbi.nlm.nih.gov/>) by basic local alignment search tool BLAST using the human cyclophilin A (hCYPA), FKBP12 (hFKBP12) and parvulins (hPAR14/hPIN1), and PTPAs (YPA1/YPA2) of *S. cerevisiae* as a query. The domains in the putative PPIase proteins were identified with CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), PFAM (<http://pfam.xfam.org>), Prosite (<https://prosite.expasy.org/>) and SMART (<http://smart.embl-heidelberg.de>) database servers. Signal peptide and transmembrane predictions were performed with SignalP4.0 (<http://www.cbs.dtu.dk/services/SignalP-4.0/>) and TMHMM ServerV2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Subcellular localization and nuclear localization signals (NLS) were predicted with LocTree3 (<https://roslab.org/services/loctree3/>) and NLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), respectively. The predicted molecular weights and the isoelectric points were determined by the compute_pi server (http://web.expasy.org/compute_pi/). The homology search was repeated 2-3 times to identify all significant matches. Pairwise percentage sequence identity and similarity were calculated using the Matrix Global Alignment Tool (MatGAT) version 2.02 using a BLOSUM50 scoring matrix. Multiple sequence alignment of amino acid sequences belonging to cyclophilins, FKBP and parvulins were performed using MUSCLE algorithm in Jalview software 2.11.1.3. with default parameters (<http://www.jalview.org/>). Secondary structure features were predicted using Jpred3 server (<http://www.compbio.dundee.ac.uk/www-jpred/>). The phylogenetic tree was constructed with the aligned PPIase sequences using the ClustalW algorithm in Mega X software and using the neighbor-joining (NJ) method with default options. For statistical reliability, the bootstrap analysis was conducted with 1000 replicates. The constructed tree files were visualized and finally drawn by Iterative Tree of Life (iTOL). The MEME (<http://meme-suite.org/tools/meme>) was used to analyze conserved and potential motifs with the parameter settings of a minimum motif width of 6, a maximum motif width of 150, and a maximum number of motifs of 15. Exon-intron organization was predicted using the Gene Structure Display Server 2.0 (GSDS 2.0) (<http://gsds.cbi.pku.edu.cn/>) by comparing the coding sequences with corresponding DNA sequences.

cDNA synthesis and quantitative Real-time PCR (qRT-PCR):

The expression of different cyclophilin, FKBP, parvulin and PTPA genes in *P. oxalicum* was analyzed by the real-time PCR by designing gene-specific primers (Supplementary Table S11). Total RNA from the harvested mycelia was isolated at different stages of growth using a Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. After removing DNA by DNaseI (Sigma-Aldrich) treatment, the RNA was quantified, and its integrity was confirmed by denaturing agarose gel electrophoresis (1.4%) followed by staining with ethidium bromide. Superscript III First-strand synthesis system kit (Invitrogen) was used to synthesize cDNA from 5 µg RNA using Random Hexamer primers according to the manufacturer's instructions. The cDNA products were diluted 10-fold prior to use for real-time PCR. The primers for qRT-PCR were designed using Primer-BLAST⁸² and primer 3 (<https://bioinfo.ut.ee/primer3-0.4.0/>). Semi-quantitative reverse transcriptase (RT)-PCR was performed to check the specificity of primers prior to the qRT-PCR. The qRT-PCR was carried out using Ariamx Real-time PCR system with Brilliant III ultra-Fast SYBR green QPCR master mix (Agilent Technologies, USA) according to manufacturer's protocol. The 10 µl qRT-PCR reaction consisted of 1X SYBR Green QPCR master mix, 60 ng of cDNA and 100 nM forward and reverse primers. The PCR programme comprised of initial incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s; annealing for 15 s at 60 °C and 20 s extension at 72 °C, followed by melt curve analysis to verify the amplification specificity. To check the contamination, dimer formation and presence of genomic DNA, no template and No reverse transcriptase controls were also included. Gene encoding actin (ACTIN) was used as a reference. The Ct values were processed by $2^{-\Delta\Delta Ct}$ method to calculate the relative mRNA levels for different genes⁸³. All analyses were performed in three biological replicates with three technical replicates. The data obtained were subjected to ANOVA.

Statistical analysis:

All the data were presented as mean \pm S.E. All the experiments were performed in triplicate unless otherwise specified. The data were analyzed by two-way analysis of variance (ANOVA) via Tukey's multiple comparison test using Graph pad prism 7 software.

Results

The halotolerant endophytic fungal strain used in the present study for expression analysis of PPlase genes was identified as *P. oxalicum*. Microscopic observations revealed fungal hyphae to be highly branched with long brush-like branched conidiophores producing phialides with a short narrow neck. Conidia were smooth-walled, cylindrical to ellipsoidal and produced in chains in long parallel columns (Fig. 1a). Though this strain was able to grow in the presence of up to 15% NaCl, the growth was substantially higher in the medium lacking salt (Fig. 1b) since the colony diameter after ten days of incubation at 30°C was higher (5.1 cm) compared to salt stress (1.2 cm). Further, relative to the unamended medium, the colonies obtained in the presence of salt were compact and depicted reduced sporulation. The mycelial fresh and dry weights of the culture were affected differently by salt stress. While the fresh weight was higher in the medium lacking salt, the mycelial dry weight was significantly greater in the presence of NaCl (Fig. 1c), which is in agreement with similar observations reported earlier for other fungi⁴⁴. However, ultrastructure studies and the estimation of compatible solutes are required to understand the mechanism responsible for the salt-induced increase in the mycelial biomass of this strain.

Bioinformatics analysis

Cyclophilins

In silico analysis revealed 237 full-length CLD-containing putative cyclophilin proteins in different species of *Penicillium* (Table 1). The number of cyclophilins in different *Penicillium* spp. range between 7–11, with the *P. oxalicum* genome encoding ten cyclophilins (Supplementary Table S1). Based on homology, the *Penicillium* cyclophilins were clustered into 12 different orthogroups viz., PenCYP01-PenCYP12 (Table 2, Supplementary Table S2a-l), which was also validated by their phylogenetic clustering (Fig. 2). Genes encoding different cyclophilins of the same orthogroup depicted conservation in their intron-exon architecture (Supplementary Fig. S1). While the genes for PenCYP09 cyclophilins (Group C) showed the absence of introns, the genes of orthogroup PenCYP06 (Group H) depicted up to seven introns (Supplementary Fig. S1). The PenCYP01, PenCYP05, PenCYP06 and PenCYP11 members were observed in all *Penicillium* spp., suggesting their essential role (Table 2). The PenCYP12 orthogroup comprises of only two cyclophilins, PcoCYP121 (121.93 kDa) and PgrCYP121 (121.9 kDa), that were observed only in *P. coprophilum* and *P. griseofulvum*, respectively (Supplementary Table S2l). Variable homology was noticed among cyclophilins of different orthogroups, with the interspecific divergence being higher in the members of PenCYP06 and PenCYP10, that showed a minimum similarity of 51.6% and 41.3%, respectively (Supplementary Table S3f, j). On the contrary, the orthogroups PenCYP01, PenCYP11 and PenCYP12 demonstrated greater conservation, with the minimum similarity being 90.7%, 84.2 % and 97.1%, respectively (Supplementary Table S3a, k, l).

The predicted molecular weights (MWs) and pIs of the *Penicillium* cyclophilins range between 17.63 kDa (*P. steckii*) to 126.82 kDa (*P. arizonense*), and 4.56 (*P. expansum*) to 9.32 (*P. steckii*), respectively (Table 1). The cyclophilins in *P. oxalicum* also showed divergence in their MWs and pIs, with the values ranging between 17.79 kDa (PoxCYP17) to 69.82 kDa (PoxCYP69), and 5.93 (PoxCYP41) to 8.87 (PoxCYP18), respectively (Supplementary Table S1). Though predominantly cytosolic, the cyclophilins in *Penicillium* were also predicted to localize to the nucleus, endoplasmic reticulum (ER) and mitochondria, highlighting functional divergence of these proteins (Table 1; Supplementary Table S2a-l). Besides cytosolic, the ER-localized (PoxCYP23) and nuclear PPlases (PoxCYP54, PoxCYP62-1, PoxCYP62-2 and PoxCYP69) were also observed in *P. oxalicum* (Supplementary Table S1). Except for PenCYP07 cyclophilins, in which the CLD ranges between 128-179 AAs, this domain's length is similar in cyclophilins of all other orthogroups (Supplementary Table S2a-l). The secondary structure of CLD, comprising of a typical β -barrel of eight antiparallel β -sheets with the two ends closed by α -helices and represented as $\beta\beta\alpha\beta\alpha\beta\beta\beta\beta\alpha\beta\beta\alpha\beta\alpha\beta$ in hCYPA (Supplementary Fig. S2)²⁴, showed conservation in cyclophilins of all orthogroups except PenCYP03, PenCYP05, PenCYP11 and PenCYP12 which either lack or contain a partial β 1 region. Based on the presence of domains other than CLD, the cyclophilins were further classified as single domain (SD) or multidomain (MD) proteins (Table 3). While seven orthogroups (PenCYP01-PenCYP06 and PenCYP09) consist of SD cyclophilins, five orthogroups (PenCYP07, PenCYP08, PenCYP10-PenCYP12) comprise of MD proteins that contain additional domains such as TPR, RRM, U-box, WD, PP2C, and GIT_SDH (Supplementary Table S2a-l). Both SD (6) and MD cyclophilins (4) were also observed in *P. oxalicum* (Supplementary Table S1). This study predicted 15 different motifs within and outside the CLD (Supplementary Fig. S1), the motif composition being conserved in different cyclophilins of the same orthogroup. Comparative analysis with hCYPA revealed that all the active site residues corresponding to Arg (55), Phe (60), Met (61), Gln (63), Ala (101), Phe (113), Trp (121), Leu (122) and His (126), essential for PPlase activity and CsA interaction, are conserved in all cyclophilins of orthogroups PenCYP03, PenCYP06, PenCYP11 and PenCYP12 (Table 4, Supplementary Fig. S2). In *P. oxalicum* also, the PoxCYP17, PoxCYP18, PoxCYP23 and PoxCYP69 proteins showed retention of all the active site residues (Supplementary Table S4). Thus, these proteins are likely to be enzymatically active and might perform different cellular functions due to their PPlase activity. On the contrary, the cyclophilins belonging to the groups PenCYP02, PenCYP04, PenCYP05 and PenCYP07-PenCYP10 exhibited several substitutions in their active site residues, with the most common being Trp (121)/His (126) replaced with other residues (Table 4, Supplementary Fig. S2). While Trp121 in hCYPA is essential for CsA binding and changes in this residue result in decreased sensitivity to this immunosuppressant, mutations in the other active site residues are known to result in alteration in the PPlase activity^{45–48}. The effect of alterations in the active site residues on PPlase activity of these cyclophilins needs further evaluation by cloning and characterizing these proteins.

The phylogenetic relationship among different cyclophilins was studied by constructing an unrooted tree based on proteins consisting of full-length or partial CLD sequences. This analysis divided the *Penicillium* cyclophilins into 11 distinct groups, A-K (Fig. 2). Interestingly, no *P. oxalicum* cyclophilin was observed in group G, suggesting that this gene might have been acquired by other species or lost from the *P. oxalicum* during the course of evolution. Similar events were implicated earlier in the evolution of plant NAC gene family also⁴⁹. A noteworthy feature of Group K, comprising of PenCYP07 orthogroup, is the presence of PcaCYP7, PexCYP8, PitCYP8, PgrCYP7, and PsoCYP7 (that contain only N-terminus CLD) along with PcaCYP33, PexCYP33, PitCYP33, PgrCYP33 and

PsoCYP33 (which possess only C-terminus CLD). It is likely that PcaCYP7, PexCYP8, PitCYP8, PgrCYP7 and PsoCYP7 might be the result of deletion of N-terminus region of CLD in PcaCYP33, PexCYP33, PitCYP33, PgrCYP33 and PsoCYP33, respectively. This speculation is supported by the fact that pairwise alignment of PcaCYP7, PexCYP8, PgrCYP7, PitCYP8, and PsoCYP7 with PcaCYP33, PexCYP33, PgrCYP33, PitCYP33, and PsoCYP33, respectively, corresponded to full-length cyclophilins that are homologous to other members of the same group (Supplementary Fig. S3). Though PgrCYP121 and PcoCYP121 were clustered in Group I, pairwise comparison prompted us to designate these proteins as a separate orthogroup PenCYP12 due to the presence of a large stretch of 950 AA residues that was not observed in other members of this group. The two proteins depicted 97% and 94% similarity in their GIT_SDH and CLD domains. Interestingly, GIT_SDH domain has not been reported yet in any of the *Penicillium* cyclophilins.

FK506-binding proteins (FKBPs)

Ninety-three putative FKBPs were identified in *Penicillium* spp. by basic local alignment search tool (BLAST) analysis using the human FKBP, hFKBP12, as a query. The hFKBP12 is the smallest member (12 kDa) of the FKBP family and contains the PPLase core domain^{50,51}. Based on similarity, these proteins were categorized into four different orthogroups viz., PenFKBP01, PenFKBP02, PenFKBP03 and PenFKBP04 (Table 2). This grouping was also supported by the phylogenetic analysis, which depicted a close relationship of these proteins within a group (Fig. 3a). All *Penicillium* spp. except *P. decumbens*, *P. occitanis* and *P. steckii* depicted four different FKBPs. While *P. decumbens* contain only two FKBPs, both *P. occitanis* and *P. steckii* consist of three each (Table 2). Interestingly, *P. antarcticum* exhibited two different PenFKBP02 proteins, PanFKBP12-1 and PanFKBP13, that are 72.1% similar and appear to be paralogous (Supplementary Table S5b). The presence of PenFKBP02 and PenFKBP03 FKBPs in all *Penicillium* spp. underlines their essential role in the cell. The number of introns in FKBP genes varies between 1 to 5, with PsuFKBP61 of the orthogroup PenFKBP01 being the only exception with seven introns (Supplementary Fig. S4). Except for PsuFKBP61, the intron-exon architecture showed conservation in the FKBP genes of the same orthogroup. The FKBPs of orthogroup PenFKBP02 showed highest similarity with hFKBP12 (58%-71.3%), followed by PenFKBP01 (13.4%-61.2%), PenFKBP03 (36.4%-51.1%), and PenFKBP04 (14.8%-16%) (Supplementary Table S5a-d). Of the different *P. oxalicum* FKBPs, the maximum similarity with hFKBP12 was observed for PoxFKBP12-1 (64.5%), followed by PoxFKBP12-2 (61.2%), PoxFKBP14 (51.1%) and PoxFKBP52 (15.1%) (Supplementary Table S5e). The similarity among different FKBPs in *P. oxalicum* ranges between 15.3% -73.6% (Supplementary Table S5e).

Interspecific variability observed in the MWs and pIs of FKBPs in each orthogroup in *Penicillium* suggests divergence (Table 5, Supplementary Table S6a-d). The MWs of FKBPs in *P. oxalicum* differ from 12.93 kDa to 52.72 kDa, with a pI range of 4.38–9.36 (Table S1). The FKBPs in *Penicillium* spp. were predicted to localize to different subcellular organelles. While members of the orthogroups PenFKBP01 and PenFKBP02 might localize to the cytosol, the PenFKBP03 and PenFKBP04 FKBPs are likely to be present in the ER and nucleus, respectively (Table 3). An ER retention sequence (KDEL) (Supplementary Fig. S5) might be responsible for the likely presence of PenFKBP03 proteins in the ER. Contrary to the PenFKBP01, PenFKBP02 and PenFKBP03 proteins, which consist of only FKBP domain, the PenFKBP04 members also exhibited a nucleoplasmic like (NPL) domain (Table 3). The FKBP domain, consisting of four to six antiparallel beta-sheets surrounding the alpha-helix and represented as $\beta\beta\beta\beta\alpha\beta\alpha\beta\alpha\beta\beta$ in hFKBP12²⁴, is conserved in all *Penicillium* FKBPs except for few members of orthogroup PenFKBP01 that lack the β 1-sheet (Supplementary Fig. S6). Of the 15 different motifs observed in *Penicillium* FKBPs, the motifs 1 and 3, part of the FKBP domain, were observed in all the proteins. (Supplementary Fig. S4). Comparison of the 13 key residues that are implicated in FK506-binding⁵² revealed that relative to hFKBP12, the members of orthogroups PenFKBP01, PenFKBP02, PenFKBP03 and PenFKBP04 showed conservation at six, eight, nine and ten positions respectively (Table 4, Supplementary Fig. S6).

Parvulins

On the basis of homology with human parvulins hPIN1 (Protein Interacting with NIMA) and hPAR14 (human parvulin 14), the *Penicillium* parvulins were grouped into two different orthogroups viz., PenPIN01 and PenPAR01, respectively (Table 2). Phylogenetic analysis also provided evidence for the evolutionary relationship of these proteins within each orthogroup (Fig. 3b). The genes encoding PenPIN01 (except PsuPIN21) and PenPAR01 showed one and two introns, respectively (Supplementary Fig. S7). Whereas, PenPAR01 proteins were observed in all *Penicillium* spp. analyzed, the PenPIN01 parvulins were not detected in *P. antarcticum*, *P. arizonense*, *P. coprophilum* and *P. solitum* (Table 2). The PenPAR01 and PenPIN01 parvulins showed 53.7%-68.5%, and 57.7%-66.7%, similarity with hPAR14 and hPIN1, respectively (Supplementary Table S7a, b). In *P. oxalicum* also, the PoxPAR14 and PoxPIN1 shared 66.9% and 65.7% similarity with their human orthologues hPAR14 and hPIN1, respectively. High similarity among members of PenPAR01 (65.4-100%) and PenPIN01 (76.5%-100%) groups implies conservation of parvulins among different species of *Penicillium* (Supplementary Table S7a, b). Except for PdePAR17 (17.67 kDa) in *P. decumbens*, the MWs of PenPAR01 proteins differed between 13.62 to 14.75 kDa, and that of PenPIN01 members between 19.30 to 21.88 kDa (Table 6). The pI values in orthogroups PenPAR01 and PenPIN01 varied between 9.41 to 9.68, and 5.72 to 6.46, respectively. The larger size of PdePAR17 is attributed to an extended N terminal 36 amino acid sequence containing mitochondrial localization signal⁵³. Except for PdePAR17, which might be a mitochondrial protein, all members of the PenPAR01 orthogroup were predicted to localize to either cytosol or nucleus. Majority of the PenPIN01 proteins, on the contrary, appeared to be nuclear, with only *P. steckii* (PstPAR13) and *P. subrubescens* (PsuPAR14) depicting localization in the cytoplasm. Contrary to the PenPAR01 parvulins, that contain only the PPLase domain, the PenPIN01 members also exhibited an additional conserved N-terminal WW domain (Table 3). The PenPAR01 and PenPIN01 parvulins contain ten different motifs, with the motifs 1, 4 and 5 present in all members. (Supplementary Fig. S7). Whereas all the ten active site residues relative to hPIN1 are conserved in PenPIN01 parvulins, only five active site residues in PenPAR02 members showed conservation relative to hPAR14 (Table 4). As observed in hPAR14 and hPIN1⁵⁴, all *Penicillium* parvulins exhibited the presence of β 1 α 1 α 2 α 3 β 2 α 4 β 3 β 4 elements in their PPLase domain (Supplementary Fig. S8a, b), suggesting that the secondary structure of these proteins is conserved across taxa. Though conservation of these proteins underlines their fundamental role in the cell, the absence of PenPIN01 members in *P. antarcticum*, *P. arizonense*, *P. coprophilum* and *P. solitum* also suggests redundancy in their functions.

Protein phosphatase 2A phosphatase activators (PTPAs)

The members of PenPTPA01 and PenPTPA02 orthogroups in *Penicillium* spp. were identified by BLAST analysis based on their similarity with their yeast orthologues YPA1 and YPA2, respectively. This analysis revealed that except for *P. nalgiovense* and *P. steckii*, which lack PTPA02 gene, all other *Penicillium* species contain both the PTPAs (Table 2, 7). Phylogenetic analysis also supported a close evolutionary relationship among proteins of each orthogroup (Fig. 3c). *In silico* studies further revealed that while all the genes encoding PenPTPA01 proteins contain two introns, the same is lacking in the PenPTPA02 genes (Supplementary Fig. S9). The YPA1 exhibited 44.3%-49.2% similarity with PenPTPA01 orthologues, compared to 53.1%-57.4% for YPA2 with PenPTPA02 members (Supplementary Table S8a, b). The molecular weights of PenPTPA01 and PenPTPA02 vary between 46.43 to 52.97 kDa, and 46.05 to 47.55 kDa, respectively, while the pI values for the two PPlases range between 5.81-7.20 and 5.84-6.44, respectively (Table 7). The PenPTPA01 and PenPTPA02 proteins in *Penicillium* spp. were predicted to localize to the cytosol, and consist of only PTPA domain of 283-331 and 293-295 amino acid residues, respectively. The two PTPA orthogroups revealed the presence of 15 different motifs, of which six (1-3, 6 and 9) are common to all members (Supplementary Fig. S9). High similarity among PenPTPA01 (69.3%-100%) and PenPTPA02 (77.1%-100%) members in *Penicillium* spp. suggests conservation, indicating an essential role for these proteins in the cell (Supplementary Table S8a, b).

Estimation of PPlase activity and expression analysis of PPlases genes in *P. oxalicum*

The total and specific PPlase activities under salt stress were significantly higher than control at all the stages of growth in *P. oxalicum* (Fig. 4a, b,c). Further, the PPlase activity under control conditions was not regulated temporally since no significant difference in the mycelial catalytic activity was observed at different growth stages. On the contrary, substantial enhancement in the specific PPlase activity was noticed between 4 and 7 days after inoculation (DAI) under salt stress that appeared to be due to induction of PPlases since decrease in total protein content during this duration was 40.3% (from 9.04 to 5.4 mg/g fresh weight) compared to 88.7% (from 8.3 to 15.68 nmol/sec/mg protein) increase in specific PPlase activity (Fig. 4c, d). FKBP and cyclophilins' contribution to PPlase activity in *P. oxalicum* was evaluated by the extent of inhibition by their specific inhibitors FK506 and CsA, respectively. Whereas PPlase activity under control conditions was almost completely inhibited by CsA at all the growth stages, the CsA-induced inhibition in the presence of salt was about 85% and 87% at 4 and 7 DAI, respectively (Fig. 4e). These observations imply that PPlase activity in the mycelia of *P. oxalicum* was predominantly contributed by the cyclophilins. However, 15% and 13% abrogation of PPlase activity by FK506 at 4 and 7 DAI under salt stress also indicated the contribution of FKBP to enzyme activity at these stages. We carried out real time-PCR analysis to further analyze the contribution of different PPlase genes to the mycelial PPlase activity. This analysis revealed that three cyclophilin (*PoxCYP18*, *PoxCYP23* and *PoxCYP41*) and two FKBP genes (*PoxFKBP12-2* and *PoxFKBP52*) were expressed at all stages of growth under both control and salt stress conditions (Fig. 5). Whereas the expression of *PoxCYP18* at 4 and 10 DAI increased significantly under salt stress, the transcript levels of *PoxCYP23*, *PoxCYP41*, *PoxFKBP12-2* and *PoxFKBP52* at all stages of growth decreased substantially. However, the transcripts corresponding to parvulins and PTPAs were not observed at any of the stages analyzed.

Discussion

The present study reports *in silico* characterization of PPlase gene families in *Penicillium*, and their regulation by salt stress in the mycelia of a halotolerant strain of *P. oxalicum*. This analysis revealed that the number of cyclophilins in *Penicillium* spp. varies between 7 to 11 (Table 1), which is comparable to that reported in other fungi^{8,40,55,56}. The cyclophilins in *Penicillium* spp. were predicted to localize to different cellular compartments. The cytoplasmic cyclophilins PenCYP01 and PenCYP05, the ER-targeted PenCYP06 and the nuclear predicted PenCYP11 were observed in all *Penicillium* spp. (Table 3), suggesting their indispensable role in the cell. The cyclophilins also play an essential role in mitochondria, with the CPR3 and CyP-D in yeast and humans, respectively, implicated in the regulation of mitochondrial permeability transition pore⁵⁷⁻⁵⁹. However, contrary to the widespread prevalence of these proteins in mitochondria of different fungi⁴⁰, our analysis revealed the presence of mitochondrial cyclophilins only in seven species of *Penicillium* (Table 1). The absence of mitochondrial cyclophilins in majority (17) of the *Penicillium* spp., therefore, needs to be validated by analyzing the CsA-inhibitable PPlase activity in these subcellular organelles.

The occurrence of different functional domains points towards the acquisition of novel roles by these cyclophilins, since domains such as TPR and WD repeats facilitate protein-protein interactions, while RRM and U-box containing proteins have been implicated in RNA stabilization and ubiquitination, respectively⁶⁰⁻⁶³. The presence of RRM and nuclear localization signal in the cyclophilins of orthogroup PenCYP08 (Table 3) suggests that these proteins may have a role in RNA processing or regulation of transcription^{64,65}. A noteworthy feature of this study was that cyclophilins with GIT_SHD2 domain (orthogroup PenCYP12) were observed only in *P. coprophilum* and *P. griseofulvum* (Supplementary Table S21). To our knowledge, the GIT_SHD2 domain has not been reported in cyclophilins of other fungi studied yet. Therefore, this novel combination of domains indicates species-specific neofunctionalization of cyclophilins in *Penicillium* and warrants in depth investigations to understand their cellular implications.

The FKBP repertoire in *Penicillium* varies from two in *P. decumbens* to five in *P. antarcticum* (Table 2), which is consistent with similar findings in other fungi⁴⁰. Further, the localization of *Penicillium* FKBP to the cytosol, ER and nucleus, and the presence of a single FKBP domain in these proteins (Table 3) is also in accordance with the earlier studies⁴⁰. The role of different FKBP in *Penicillium* has not been analyzed yet but studies with *Schizosaccharomyces pombe* demonstrated that though not essential, the SpFKBP12 plays a vital role in the early steps of sexual development pathway⁶⁶. Since SpFKBP12 is orthologous to PenFKBP02 members, the latter may also be performing a similar role in the cell which needs to be confirmed by further experimentation. The ER-localized FKBP of the orthogroup PenFKBP03 (Table 3) showed a high degree of homology (66.9%) with human FKBP13 (Supplementary Table S9), a membrane-associated protein localized to the lumen of the ER⁶⁷⁻⁶⁹. As suggested for hFKBP13⁷⁰, the PenFKBP03 FKBP may also be involved in protein folding in the ER that is imperative for the survival of the cells and explains the presence of these proteins in all *Penicillium* spp. On the contrary, the ability of *P. decumbens*, *P. occitanis* and *P. steckii* to complete growth and development despite lacking either PenFKBP01 or both PenFKBP01 and PenFKBP04 members (*P. decumbens*) (Table 2) indicates redundancy in the functions of these proteins. Owing to the NPL domain (Table 3), the PenFKBP04 Group FKBP in *Penicillium* might be involved in nucleosome assembly and regulation of gene expression, as demonstrated for the NPL containing yeast FKBP, Fpr4^{71,72}, with

which these proteins show considerable similarity (52.5%-56.4%) (Supplementary Table S10). The existence of NPL domain suggests the acquisition of novel roles by these proteins that may be enabling the cells to respond to different developmental and environmental cues.

Parvulins have been implicated in post-phosphorylation control of diverse cellular processes such as cell division, gene expression, immune response, etc.⁷³⁻⁷⁵. The identification of parvulins in *Penicillium* with and without WW domain (Table 3) supports the earlier findings in *E. coli*, yeast and *Arabidopsis*^{8,76}. Though the N-terminal WW domain in PIN1-type parvulins facilitates specific binding to phosphorylated Thr/Ser-Pro motifs and their subsequent *cis-trans* isomerization by the C-terminal PPlase domain⁷⁷, the PIN1-type PPlases in *Arabidopsis* and *Malus domestica* plants, despite lacking this domain, exhibited no difference in substrate specificity⁷⁸. Therefore, further studies are required to understand the significance of the WW domain in regulating the parvulin activities in *Penicillium*.

The occurrence of genes encoding PenPTPA01 and PenPTPA02 in all *Penicillium* spp., except *P. nalgiovense* and *P. steckii* that lack the PenPTPA02 proteins (Table 2), signifies their indispensable role in the cell. These proteins may be involved in the activation of PP2A-like phosphatases, as reported for their yeast orthologues YPA1 and YPA2⁶. Though information about the active site residues required for PPlase activity in PTPAs is elusive, deletion of a conserved domain 208-GVWGLD-213 in YPA1 resulted in about 400-fold attenuation of phosphotyrosyl phosphatase activation reaction of PP2A⁶. Since the same amino acid stretch is also present in the PenPTPA01 and PenPTPA02 members in all *Penicillium* spp. (Supplementary Fig. 10), these proteins may likely have PPlase activity. Cloning and characterization of these proteins are, nevertheless, required to validate this speculation.

Analysis of PPlase activity and expression of PPlase genes

Biochemical investigations revealed significantly higher PPlase activity under salt stress in the halotolerant *P. oxalicum*, which was predominantly inhibited by CsA, underscoring the contribution of cyclophilins (Fig. 4a-e). Of the three cyclophilins (*PoxCYP18*, *PoxCYP23* and *PoxCYP41*) and two FKBP genes (*PoxFKBP12-2* and *PoxFKBP52*) expressed in the mycelia, the salt-induced increase was observed only for *PoxCYP18* (Fig. 5), implying its contribution to the mycelial PPlase activity. Though the maximum increase in transcript levels of *PoxCYP18* was observed at 4 DA1, the PPlase activity was highest at 10 DA1. It is likely that this gene may be regulated at the post-transcriptional level and/or the expressed protein is highly stable and gets accumulated with time, thereby, leading to enhanced activity at later stages of growth. Estimation of the PPlase proteins by immunoblotting is, nonetheless, required to understanding the molecular basis of this observation. Since protection by cyclophilins and FKBP against stress-induced damage to the cell is attributed to their PPlase activity and/or chaperone functions⁷⁹, further biochemical characterization of these proteins in *Penicillium* is imperative to elucidate their precise mechanism of action. Although expression of other PPlases was not observed at the stages studied, their role in specific developmental processes and adaptation to other stresses cannot be ruled out as previous studies have demonstrated the role of these genes in the regulation of several developmental process⁷⁹. Further studies on the expression of PPlase genes at different developmental stages are required to enhance our understanding of the role of these genes in halotolerance of *P. oxalicum*.

Conclusions

To conclude, this study has shown that the number of genes encoding PPlases varies between 7-11, 2-5, 1-2 and 1-2 for cyclophilins, FKBP, parvulins and PTPAs, respectively, in different species of *Penicillium*. Though cyclophilins, FKBP and parvulins have been characterized earlier in several fungal species, this is the first study to characterize all the PPlases in *Penicillium* spp. Despite conservation of the secondary structure of the CLD and FKBP domains, the cyclophilins and FKBP in *Penicillium* spp. have undergone divergence by the acquisition of novel domains such as PP2C and GIT_SDH, implying neofunctionalization. In addition to the cytoplasm, the localization of PPlases in *Penicillium* to other subcellular compartments viz., ER, mitochondria and nucleus, suggests their specific roles. This study further demonstrated that the mycelial PPlase activity in a halotolerant strain of *P. oxalicum* is induced significantly under salt stress and is primarily contributed by the cyclophilins, signifying the role of these genes in stress response.

Declarations

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Tables

Table 1: Genome-wide analysis of cyclophilin proteins in different *Penicillium* spp.

S. No.	<i>Penicillium</i> spp.	Genes	Proteins	AA residues	MW (kDa)	pI	SD	MD	Subcellular localization
1	<i>P. antarcticum</i>	09	09	162-627	17.76-69.22	5.59-8.26	05	04	C, ER, N
2	<i>P. arizonense</i>	10	10	162-1183	17.77-126.82	5.32-8.70	06	04	C, ER, N
3	<i>P. brasilianum</i>	10	10	162-657	17.85-72.46	5.28-8.93	06	04	C, ER, N
4	<i>P. camemberti</i>	10	10	73-627	17.73-69.56	5.5-8.450	07	03	C, ER, N
5	<i>P. chrysogenum</i>	10	10	162-627	17.70-69.51	5.76-8.59	07	03	C, ER, N
6	<i>P. coprophilum</i>	08	08	162-1110	17.77-121.93	5.78-8.61	05	03	C, ER, N
7	<i>P. decumbens</i>	07	07	159-629	17.76-69.56	5.93-9.13	06	01	C, ER, M, N
8	<i>P. digitatum</i>	11	11	162-627	17.67-69.60	4.68-8.60	07	04	C, ER, N
9	<i>P. expansum</i>	10	10	73-627	17.72-69.48	4.56-8.91	07	03	C, ER, N
10	<i>P. flavigenum</i>	09	09	162-627	17.71-69.48	5.49-9.14	06	03	C, ER, M, N
11	<i>P. freii</i>	11	11	162-627	17.70-69.52	5.42-8.96	07	04	C, ER, M, N
12	<i>P. griseofulvum</i>	10	10	73-1108	17.74-121.89	5.68-8.63	06	04	C, ER, N
13	<i>P. italicum</i>	10	10	73-627	17.70-69.55	4.66-8.40	07	03	C, ER, N
14	<i>P. nalgioense</i>	11	11	162-627	17.68-69.61	4.63-8.96	07	04	C, ER, M, N
15	<i>P. nordicum</i>	11	11	162-627	17.90-69.30	5.42-8.96	07	04	C, ER, M, N
16	<i>P. occitanis</i>	10	10	162-631	17.69-70.39	4.72-8.63	06	04	C, ER, N
17	<i>P. oxalicum</i>	10	10	162-627	17.79-69.82	5.93-8.87	06	04	C, ER, N
18	<i>P. polonicum</i>	08	08	162-627	17.69-69.49	5.54-7.93	06	02	C, ER, N
19	<i>P. roqueforti</i>	11	11	162-627	17.74-69.41	5.65-8.89	07	04	C, ER, N
20	<i>P. rubens</i>	11	11	162-627	17.70-69.51	5.76-8.59	07	04	C, ER, N
21	<i>P. solitum</i>	10	10	73-627	17.72-69.52	5.49-8.45	07	03	C, ER, N
22	<i>P. steckii</i>	11	11	161-629	17.63-69.81	5.73-9.32	07	04	C, ER, M, N
23	<i>P. subrubescens</i>	10	10	162-629	17.87-69.57	5.48-8.89	06	04	C, ER, N
24	<i>P. vulpinum</i>	09	09	162-627	17.76-69.47	5.76-8.95	06	03	C, ER, M, N

AA: amino acids; C: cytoplasm; ER: endoplasmic reticulum; kDa: kilodalton; M: mitochondria; MD: multi domain; MW: molecular weight; N: nucleus; pI: isoelectric point; SD: single domain.

Table 2: Representation of cyclophilin, FK506-binding protein (FKBP), parvulin, and protein phosphatase 2A activator (PTPA) orthogroups in different *Penicillium* spp.

S. No.	<i>Penicillium</i> spp.	Cyclophilins									
		Cyclophilins	PenCYP01	PenCYP02	PenCYP03	PenCYP04	PenCYP05	PenCYP06	PenCYP07	PenCYP08	Pen
1	<i>P. antarcticum</i>	9	P	O	P	O	P	P	P	P	P
2	<i>P. arizonense</i>	10	P	O	P	P	P	P	P	P	P
3	<i>P. brasilianum</i>	10	P	P	P	O	P	P	P	P	P
4	<i>P. camemberti</i>	10	P	P	P	P	P	P	O	P	P
5	<i>P. chrysogenum</i>	10	P	P	P	P	P	P	O	P	P
6	<i>P. coprophilum</i>	8	P	P	O	P	P	P	O	O	O
7	<i>P. decumbens</i>	7	P	P	P	P	P	P	O	O	O
8	<i>P. digitatum</i>	11	P	P	P	P	P	P	P	P	P
9	<i>P. expansum</i>	10	P	P	P	P	P	P	O	P	P
10	<i>P. flavigenum</i>	9	P	P	P	P	P	P	O	P	O
11	<i>P. freii</i>	11	P	P	P	P	P	P	P	P	P
12	<i>P. griseofulvum</i>	10	P	P	O	P	P	P	O	P	P
13	<i>P. italicum</i>	10	P	P	P	P	P	P	O	P	P
14	<i>P. nalgiovense</i>	11	P	P	P	P	P	P	P	P	P
15	<i>P. nordicum</i>	11	P	P	P	P	P	P	P	P	P
16	<i>P. occitanis</i>	10	P	P	P	O	P	P	P	P	P
17	<i>P. oxalicum</i>	10	P	P	P	O	P	P	P	P	P
18	<i>P. polonicum</i>	8	P	P	O	P	P	P	O	O	P
19	<i>P. roqueforti</i>	11	P	P	P	P	P	P	P	P	P
20	<i>P. rubens</i>	11	P	P	P	P	P	P	P	P	P
21	<i>P. solitum</i>	10	P	P	P	P	P	P	O	P	P
22	<i>P. steckii</i>	11	P	P	P	P	P	P	P	P	P
23	<i>P. subrubescens</i>	10	P	P	P	O	P	P	P	P	P
24	<i>P. vulpinum</i>	9	P	P	P	P	P	P	O	P	O

O: absent; P: present; *: two copies

Table 3: Domain architecture and localization of different orthogroups of cyclophilins, FK506-binding proteins (FKBPs), parvulins and protein phosphatase 2A activators (PTPAs) in *Penicillium* spp.

	Orthogroups	Proteins	SD	MD	Domains	Localization
Cyclophilins	PenCYP01	24	24	0	CLD (24)	C
	PenCYP02	22	22	0	CLD (22)	C
	PenCYP03	21	21	0	CLD (21)	C (14), M (7)
	PenCYP04	19	19	0	CLD (19)	C
	PenCYP05	24	24	0	CLD (24)	C
	PenCYP06	24	24	0	CLD (24)	ER
	PenCYP07	13	0	13	CLD+TPR (13)	C
	PenCYP08	21	0	21	CLD+RRM (21)	N
	PenCYP09	20	20	0	CLD (20)	N
	PenCYP10	23	0	23	CLD+U-box (22)	N
					CLD+U-box + PP2C (1)	
	PenCYP11	24	0	24	CLD+WD REPEAT (24)	N
PenCYP12	2	0	2	CLD+GIT_SDH (2)	C	
FKBPs	PenFKBP01	21	21	0	FKBP (21)	C
	PenFKBP02	25	25	0	FKBP (21)	C
	PenFKBP03	24	24	0	FKBP (21)	ER
	PenFKBP04	23	0	23	FKBP+NPL (23)	N
PARs	PenPAR01	24	24	0	PPlase (24)	C (14), M (1), N (9)
	PenPIN01	20	0	20	PPlase+WW (20)	C (2), N (18)
PTPAs	PenPTPA01	24	24	0	PTPA (24)	C
	PenPTPA02	22	22	0	PTPA (22)	C

C: cytoplasm; CLD: cyclophilin-like domain; ER: endoplasmic reticulum; FKBP: FK506 binding protein; GIT_SDH: Spa2 homology domain (SHD) of GIT {G-protein-coupled receptor (GPCR)-kinase-interacting protein}; M: mitochondria; MD: multidomain; N: nucleus; NPL: nucleoplasm like domain; PP2C: Protein phosphatase 2C; PTPA: protein phosphatase 2A activator; RRM: RNA recognition motif; SD: single domain; TPR: tetratricopeptide repeat; U-box: U-box domain; WD: tryptophan-aspartate repeats; WW: Domain with 2 conserved tryptophan residues.

Table 4: Conservation of the active site residues, with respect to the human orthologues hCYP, hFKBP12 and hPIN1/hPAR14, respectively, in the cyclophilins, FK506-binding proteins (FKBPs) and parvulins of different orthogroups in different *Penicillium* spp.

Cyclophilins										
Orthogroups	Conserved ASRs	ARG (R)	PHE (F)	MET (M)	GLN (Q)	ALA (A)	PHE (F)	TRP (W)	LEU (L)	HIS (H)
		55	60	61	63	101	113	121	122	126
PenCYP01	8	R	F	M	Q	A	F/Y	W	L	H
PenCYP02	8	R	F	M	Q	A	F	F	L	H
PenCYP03	9	R	F	M	Q	A	F	W	L	H
PenCYP04	8	R	F	M	Q	A	F	H	L	H
PenCYP05	7	R	F	M	Q	A	F	H	L	S
PenCYP06	9	R	F	M	Q	A	F	W	L	H
PenCYP07	8	R	F	M	Q	A	F	H	L	H
PenCYP08	5	R	F	T/S	Q	A	I/L	Y	L	A
PenCYP09	5	R	F	V	Q	A	F	R	L/M	C
PenCYP10	8	R	F	M	Q	A	F	H	L	H
PenCYP11	9	R	F	M	Q	A	F	W	L	H
PenCYP12	9	R	F	M	Q	A	F	W	L	H

FKBPs														
Orthogroups	Conserved ASRs	TYR (Y) 27	PHE (F) 37	ASP (D) 38	ARG (R) 43	PHE (F) 47	PHE (F) 49	Gln (Q) 54	GLU (E) 55	ILE (I) 57	TRP (W) 60	TYR (Y) 83	HIS (H) 88	PHE (F) 100
PenFKBP01	6	Y	F	D	P	F/L	V	G	K/Q	I/-	W	Y	-	F
PenFKBP02	8	Y	F	D	R	F/L	S/T	G	R	I	W	Y	F/Y	F
PenFKBP03	9	Y	F	D	R	L	F	G	R	I	W	Y	I/V/M	F
PenFKBP04	10	Y	F	D	K	F	F	G	E	I	W	Y	L	F

Parvulins											
Parvulins	Conserved ASRs	HIS (H) 59	LYS (K) 63	ARG (R) 68	ARG (R) 69	CYS (C)/ ASP (D)* 113	LEU (L) 122	MET (M) 130	PHE (F) 134	SER (S)/ PHE (F)* 154	HIS (H) 157
PenPAR01 (vs hPAR14)	5	H	#	#	#	D	L	L	F	F/H	H
PenPIN01 (vs hPIN1)	10	H	K	R	R	C	L	M	F	S	H

*: residues present in hPAR14; #: residues absent in hPAR14

Table 5: Genome-wide analysis of FK506-binding proteins (FKBPs) in different *Penicillium* spp.

S. No.	<i>Penicillium spp.</i>	Genes	Proteins	AA residues	MW (kDa)	pI	SD	MD	Subcellular localization
1	<i>P. antarcticum</i>	05	05	115-478	12.33-51.91	4.34-9.36	04	01	C, ER, N
2	<i>P. arizonense</i>	04	04	121-479	12.89-52.05	4.34-9.36	03	01	C, ER, N
3	<i>P. brasilianum</i>	04	04	121-478	12.87-51.85	4.35-9.36	03	01	C, ER, N
4	<i>P. camemberti</i>	04	04	121-487	12.92-53.24	4.40-9.36	03	01	C, ER, N
5	<i>P. chrysogenum</i>	04	04	121-488	12.90-53.24	4.38-9.36	03	01	C, ER, N
6	<i>P. coprophilum</i>	04	04	122-487	12.88-53.22	4.39-9.52	03	01	C, ER, N
7	<i>P. decumbens</i>	02	02	122-130	13.09-14.15	5.59-6.72	02	00	C, ER
8	<i>P. digitatum</i>	04	04	121-497	12.98-54.37	4.49-9.36	03	01	C, ER, N
9	<i>P. expansum</i>	04	04	121-494	12.95-54.12	4.44-9.36	03	01	C, ER, N
10	<i>P. flavigenum</i>	04	04	121-486	12.90-53.04	4.37-9.36	03	01	C, ER, N
11	<i>P. freii</i>	04	04	109-489	11.53-53.43	4.42-9.43	03	01	C, ER, N
12	<i>P. griseofulvum</i>	04	04	121-491	12.90-53.56	4.33-9.52	03	01	C, ER, N
13	<i>P. italicum</i>	04	04	121-483	12.98-52.83	4.83-9.36	03	01	C, ER, N
14	<i>P. nalgioense</i>	04	04	121-489	12.87-53.25	4.35-9.36	03	01	C, ER, N
15	<i>P. nordicum</i>	04	04	121-491	12.95-53.58	4.43-9.36	03	01	C, ER, N
16	<i>P. occitanis</i>	03	03	119-478	12.77-52.10	4.33-6.57	02	01	C, ER, N
17	<i>P. oxalicum</i>	04	04	121-484	12.93-52.72	4.38-9.36	03	01	C, ER, N
18	<i>P. polonicum</i>	04	04	121-489	12.92-53.44	4.42-9.36	03	01	C, ER, N
19	<i>P. roqueforti</i>	04	04	121-475	12.89-51.70	4.43-9.36	03	01	C, ER, N
20	<i>P. rubens</i>	04	04	121-488	12.90-53.24	4.38-9.36	03	01	C, ER, N
21	<i>P. solitum</i>	04	04	121-492	12.92-53.85	4.40-9.36	03	01	C, ER, N
22	<i>P. steckii</i>	03	03	123-475	13.17-51.56	4.31-7.09	02	01	C, ER, N
23	<i>P. subrubescens</i>	04	04	121-553	12.87-61.22	4.36-6.06	02	02	C, ER, N
24	<i>P. vulpinum</i>	04	04	121-491	12.89-53.64	4.43-9.40	03	01	C, ER, N

AA: amino acids; C: cytoplasm; ER: endoplasmic reticulum; kDa: kilodalton; MD: multi domain; MW: molecular weight; N: nucleus; pI: isoelectric point; SD: single domain.

Table 6: Genome-wide analysis of parvulins in different species of *Penicillium*.

S. No.	<i>Penicillium</i> spp.	Orthogroup PenPAR01					Orthogroup PenPIN01					
		Protein name	Protein accession no.	Total AAs/ (Rotamase domain)	MW (kD)	pl	Loc	Protein name	Protein accession no.	Total AAs/ (WW domain)/ (PPlase domain)	MW (kDa)	pl
1	<i>P. antarcticum</i>	PanPAR13	OQD87118.1	127 / (35-127)	13.62	9.52	N	-	-	-	-	-
2	<i>P. arizonense</i>	ParPAR13	XP_022493562.1	127 / (35-127)	13.64	9.52	C	-	-	-	-	-
3	<i>P. brasilianum</i>	PbrPAR14	CEJ54268.1	130 / (38-130)	14.17	9.45	N	PbrPIN19	CEJ61044.1	176 / (5-38) / (67-175)	19.44	6.1
4	<i>P. camemberti</i>	PcaPAR13	CRL17938.1	129 / (37-129)	13.85	9.49	C	PcaPIN19	CRL25274.1	176 / (5-38) / (67-175)	19.58	5.7
5	<i>P. chrysogenum</i>	PchPAR13	KZN91971.1	129 / (37-129)	13.80	9.49	N	PchPIN19	KZN92659.1	176 / (5-38) / (67-175)	19.55	5.8
6	<i>P. coprophilum</i>	PcoPAR13	OQE46467.1	129 / (37-129)	13.74	9.49	C	-	-	-	-	-
7	<i>P. decumbens</i>	PdePAR17	OQD75108.1	129 / (70-162)	17.67	9.68	M	PdePIN19	OQD67334.1	176 / (5-38) / (67-175)	19.52	6.3
8	<i>P. digitatum</i>	PdiPAR13	XP_014531496.1	129 / (37-129)	13.79	9.49	C	PdiPIN19	XP_014532624.1	176 / (5-38) / (67-175)	19.56	5.7
9	<i>P. expansum</i>	PexPAR13	XP_016601110.1	129 / (37-129)	13.79	9.49	C	PexPIN19	XP_016603179.1	176 / (5-38) / (67-175)	19.62	5.7
10	<i>P. flavigenum</i>	PflPAR13	OQE30197.1	129 / (37-129)	13.79	9.49	C	PflPIN19	OQE19709.1	176 / (5-38) / (67-175)	19.55	5.8
11	<i>P. freii</i>	PfrPAR13	KUM64167.1	128 / (36-128)	13.66	9.52	C	PfrPIN19	KUM58880.1	176 / (5-38) / (67-175)	19.60	5.7
12	<i>P. griseofulvum</i>	PgrPAR13	KXG47026.1	129 / (37-129)	13.79	9.49	C	PgrPIN20	KXG54614.1	181 / (5-38) / (68-175)	20.13	5.9
13	<i>P. italicum</i>	PitPAR13	KG071764.1	129 / (37-129)	13.79	9.49	C	PitPIN19	KG073822.1	176 / (5-38) / (67-175)	19.52	5.7
14	<i>P. nalgiovense</i>	PnaPAR13	OQE86291.1	129 / (37-129)	13.77	9.49	N	PnaPIN19	OQE93697.1	174 / (5-38) / (67-174)	19.30	5.8
15	<i>P. nordicum</i>	PnoPAR13	KOS48877.1	128 / (36-128)	13.67	9.52	C	PnoPIN19	KOS38539.1	176 / (5-38) / (67-175)	19.56	5.7
16	<i>P. occitanis</i>	PocPAR14	PCH00892.1	133 / (37-133)	14.75	9.48	N	PocPIN21	PCH00362.1	194 / (6-39) / (67-174)	21.88	6.4
17	<i>P. oxalicum</i>	PoxPAR14	EPS27836.1	128 / (36-128)	14.00	9.57	N	PoxPIN19	EPS29250.1	175 / (5-38) / (67-174)	19.43	5.9
18	<i>P. polonicum</i>	PpoPAR13	OQD72015.1	128 / (36-128)	13.66	9.52	C	PpoPIN19	OQD71617.1	176 / (5-38) / (67-175)	19.59	5.7
19	<i>P. roqueforti</i>	ProPAR13	CDM33780.1	129 / (37-129)	13.79	9.49	C	ProPIN19	CDM27042.1	176 / (5-38) / (67-175)	19.59	5.7
20	<i>P. rubens</i>	PruPAR13	XP_002562081.1	129 / (37-129)	13.80	9.49	N	PruPIN19	KAF3031078.1	176 / (5-38) / (67-175)	19.55	5.8

21	<i>P. solitum</i>	PsoPAR13	OQD86406.1	129 / (37-129)	13.77	9.41	C	-	-	-	-	-
22	<i>P. steckii</i>	PstPAR13	OQE27721.1	128 / (36-128)	13.77	9.49	N	PstPIN21	OQE20301.1	196 / (26-59) / (88-195)	21.82	5.9
23	<i>P. subrubescens</i>	PsuPAR14	OKO93703.1	129 / (37-129)	14.17	9.57	N	PsuPIN21	OKP10946.1	194 / (10-43) / (72-181)	21.40	6.2
24	<i>P. vulpinum</i>	PvuPAR13	OQE10874.1	129 / (37-129)	13.79	9.49	C	PvuPIN19	OQE02824.1	177 / (5-38) / (68-176)	19.61	5.9
AA: amino acids; C: cytoplasm; kDa: kilodalton; Loc: localization; M: mitochondria; MW: molecular weight; N: nucleus; pI: isoelectric point; WW: Domain with 2 conserved tryptophan residues.												

Table 7: Genome-wide analysis of protein phosphatase 2A activators (PTPAs) in different species of *Penicillium*.

S. No.	Orthogroup PenPTPA01							Orthogroup PenPTPA02				
	<i>Penicillium</i> spp.	Protein name	Protein accession no.	Total AAs / (PTPA domain)	MW (kDa)	pI	Loc	Protein name	Protein accession no.	Total AAs / (PTPA domain)	MW (kDa)	pI
1	<i>P. antarcticum</i>	PanPTPA52	OQD82836.1	484 / (25-356)	52.43	6.43	C	PanPTPA46	OQD82187.1	415 / (54-347)	46.05	6.
2	<i>P. arizonense</i>	ParPTPA52	XP_022485488.1	484 / (25-356)	52.33	6.38	C	ParPTPA46	XP_022491893.1	415 / (54-349)	46.13	6.
3	<i>P. brasilianum</i>	PbrPTPA51	OOQ86678.1	480 / (25-356)	51.87	5.97	C	PbrPTPA46	OOQ83450.1	421 / (59-354)	46.79	6.
4	<i>P. camemberti</i>	PcaPTPA52	CRL24247.1	485 / (22-352)	52.54	6.22	C	PcaPTPA46	CRL22781.1	419 / (58-353)	46.37	6.
5	<i>P. chrysogenum</i>	PchPTPA52	KZN85308.1	487 / (24-354)	52.96	6.85	C	PchPTPA46	KZN84036.1	419 / (58-353)	46.30	6.
6	<i>P. coprophilum</i>	PcoPTPA51	OQE46369.1	476 / (24-354)	51.84	6.61	C	PcoPTPA46	OQE41359.1	420 / (59-354)	46.54	6.
7	<i>P. decumbens</i>	PdePTPA51	OQD67240.1	473 / (25-356)	51.20	5.81	C	PdePTPA46	OQD78624	415 / (57-351)	46.03	6.
8	<i>P. digitatum</i>	PdiPTPA51	XP_014532126.1	474 / (22-352)	51.49	7.20	C	PdiPTPA46	XP_014536235.1	419 / (58-353)	46.34	6.
9	<i>P. expansum</i>	PexPTPA52	XP_016598742.1	485 / (22-352)	52.82	6.85	C	PexPTPA46	XP_016596123.1	419 / (58-353)	46.31	6.
10	<i>P. flavigenum</i>	PflPTPA52	OQE29046.1	487 / (24-354)	52.90	6.43	C	PflPTPA46	OQE18147.1	419 / (58-353)	46.26	6.
11	<i>P. freii</i>	PfrPTPA52	KUM61112.1	485 / (22-352)	52.80	7.10	C	PfrPTPA46	KUM59294.1	419 / (58-353)	46.39	6.
12	<i>P. griseofulvum</i>	PgrPTPA52	KXG48326.1	487 / (24-354)	52.78	6.38	C	PgrPTPA46	KXG49099.1	419 / (58-353)	46.30	6.
13	<i>P. italicum</i>	PitPTPA52	KGO74783.1	485 / (22-352)	52.97	6.85	C	PitPTPA46	KGO75264.1	419 / (58-353)	46.46	6.
14	<i>P. nalgiovense</i>	PnaPTPA52	OQE84955.1	488 / (24-354)	52.93	6.61	C	-	-	-	-	-
15	<i>P. nordicum</i>	PnoPTPA46-1	KOS46416.1	431 / (15-298)	46.43	6.33	C	PnoPTPA46-2	KOS43152.1	419 / (58-353)	46.60	6.
16	<i>P. occitanis</i>	PocPTPA49	PCH02323.1	449 / (19-350)	49.74	6.08	C	PocPTPA47	PCG91093.1	424 / (61-354)	47.55	5.
17	<i>P. oxalicum</i>	PoxPTPA51	EPS34045.1	478 / (25-356)	51.78	5.97	C	PoxPTPA46	EPS25240.1	422 / (60-355)	46.80	5.
18	<i>P. polonicum</i>	PpoPTPA52	OQD61354.1	485 / (22-352)	52.68	6.28	C	PpoPTPA46	OQD61354.1	419 / (58-353)	46.35	6.
19	<i>P. roqueforti</i>	ProPTPA52	CDM34519.1	482 / (23-353)	52.40	6.19	C	ProPTPA46	CDM28648.1	417 / (58-353)	46.14	6.
20	<i>P. rubens</i>	PruPTPA52	XP_002557947.1	487 / (24-354)	52.96	6.85	C	PruPTPA46	XP_002559612.1	419 / (58-353)	46.30	6.
21	<i>P. solitum</i>	PsoPTPA52	OQE03232.1	485 / (22-352)	52.76	6.23	C	PsoPTPA46	OQD95247.1	419 / (22-352)	46.38	6.
22	<i>P. steckii</i>	PstPTPA52	OQE16418.1	483 / (25-356)	52.62	5.87	C	-	-	-	-	-
23	<i>P. subrubescens</i>	PsuPTPA51	OKP02397.1	479 / (25-356)	51.86	6.34	C	PsuPTPA47	OKP06461.1	420 / (58-353)	47.00	6.
24	<i>P. vulpinum</i>	PvuPTPA52	OQE05701.1	485 / (24-354)	52.88	6.33	C	PvuPTPA46	OQE00347.1	419 / (58-353)	46.48	6.

AA: amino acids; C: cytoplasm; kDa: kilodalton; Loc: localization; MW: molecular weight; pI: isoelectric point.

Figures

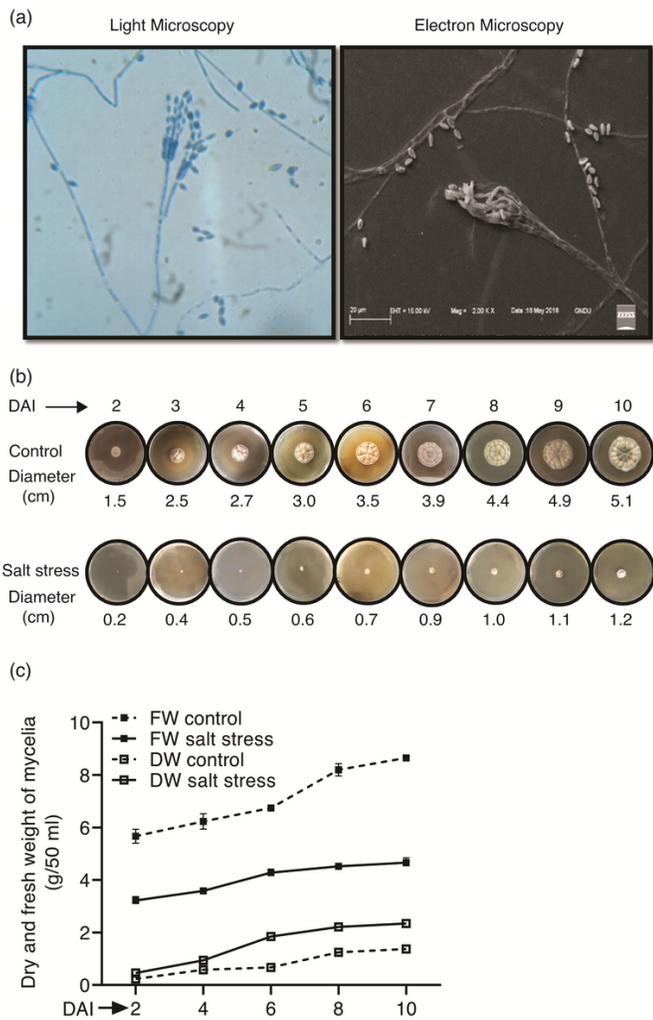


Figure 1

(a) Light and electron microscopic observations of *Penicillium oxalicum* fungal hyphae. (b) Growth pattern of *P. oxalicum* on solid medium in the presence (lower lane) or absence (upper lane) of salt stress (15% NaCl). (c) Effect of salt stress (15% NaCl) on fresh and dry weights of mycelia at different growth stages in *P. oxalicum*. The values depict the mean of three biological replicates \pm standard error. (DAI: days after inoculation).

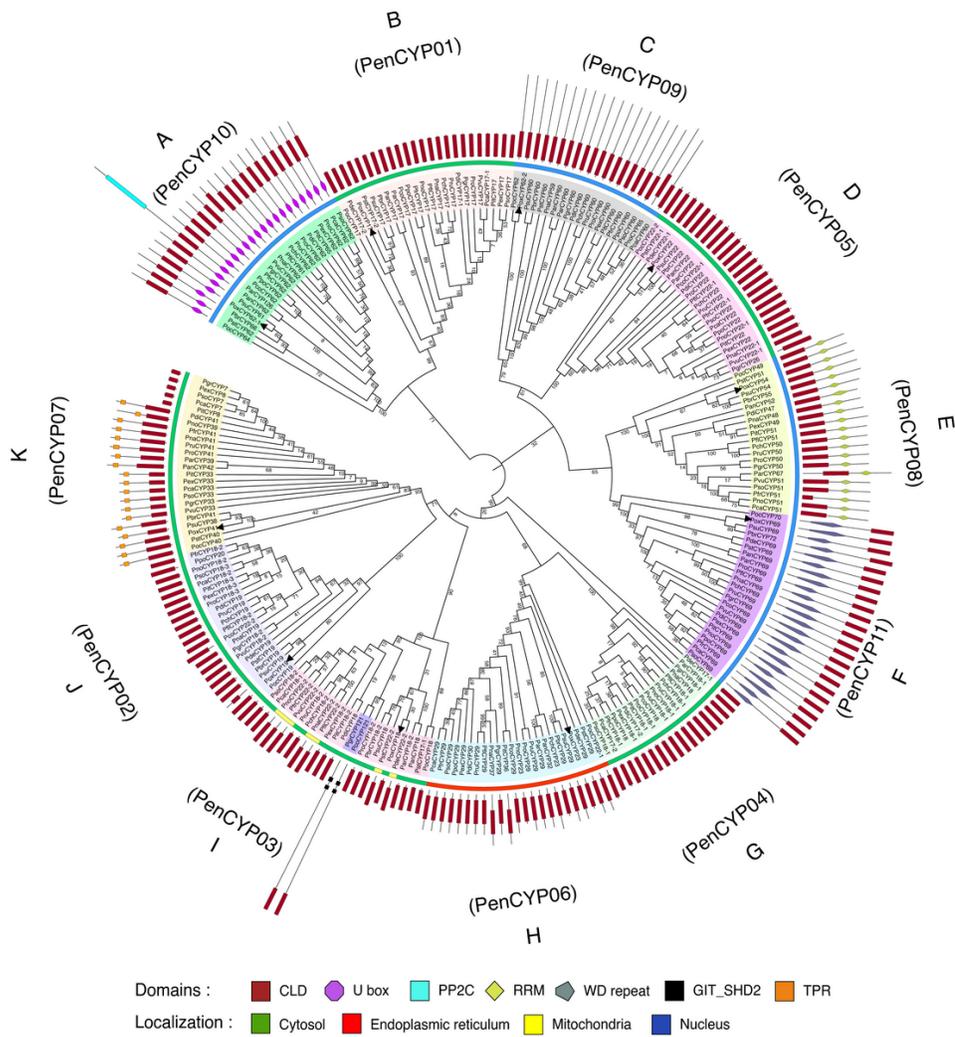


Figure 2

The phylogenetic relationship of different *Penicillium* cyclophilins. The unrooted tree was generated using the neighbor-joining method in MEGA X. Bootstrap values from 1000 replicates are indicated at each branch. Localization of each member is represented in different colors. Domain architecture of each protein is also shown in the outermost layer. Black triangles represent the *Penicillium oxalicum*.

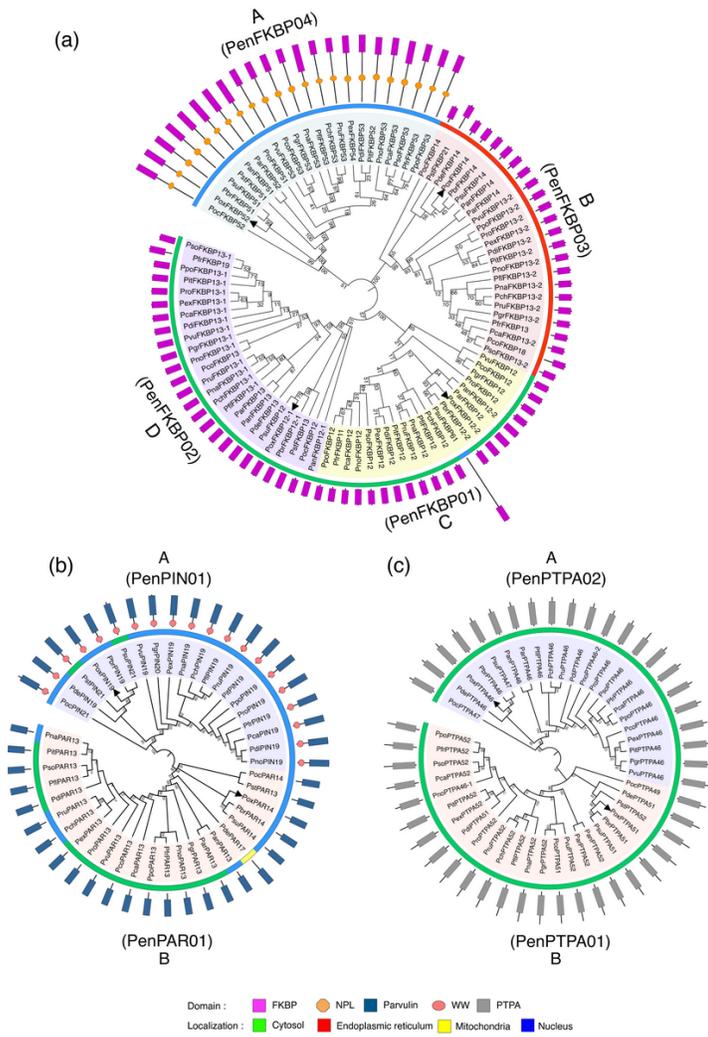


Figure 3

The phylogenetic relationship of different *Penicillium* FKBP (a), parvulins (b), and protein phosphatase 2A phosphatase activators (PTPAs) (c). The unrooted tree was generated using the neighbor-joining method in MEGA X. Bootstrap values from 1000 replicates are indicated at each branch. Localization of each member is represented in different colors. Domain architecture of each protein is also depicted in the outermost layer. Black triangles represent the *Penicillium* oxalicum.

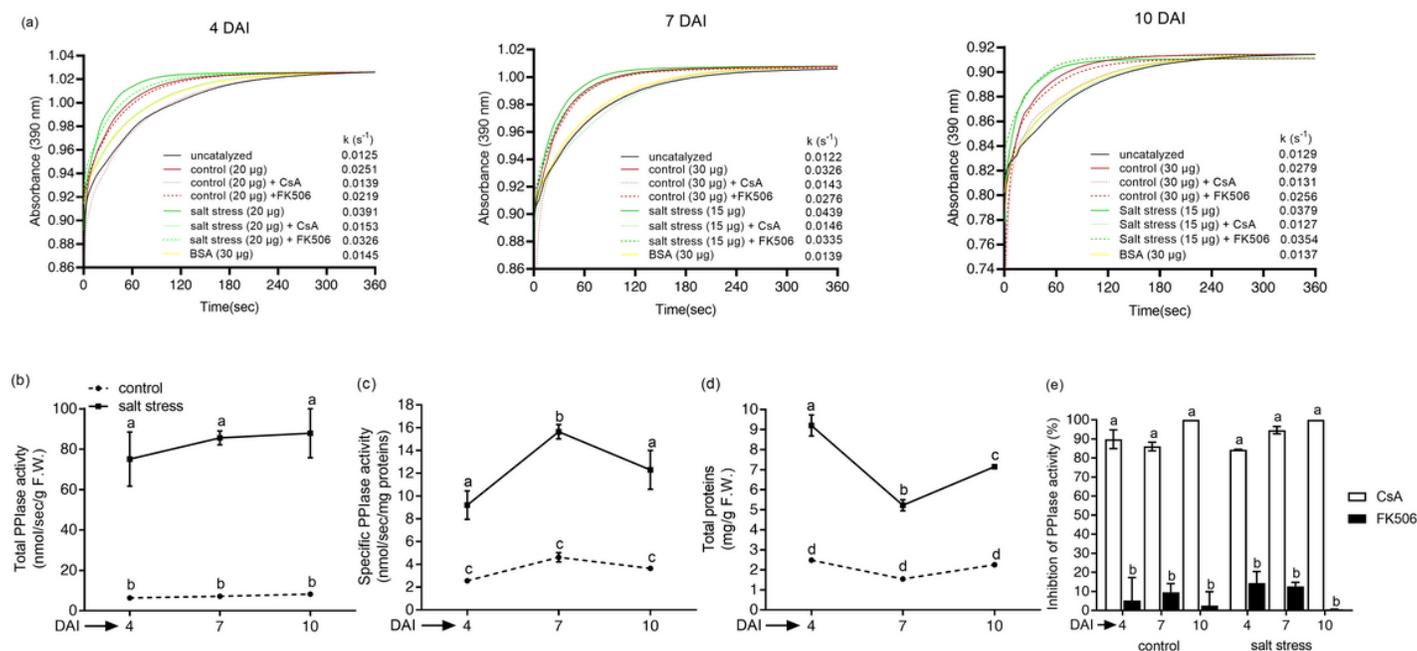


Figure 4

(a) Representative spectra showing the effect of salt stress (15% NaCl) on the mycelial peptidyl-prolyl cis-trans isomerase (PPIase) activity of *Penicillium oxalicum* at 4, 7 and 10 days after inoculation (DAI). The PPIase activity in the crude mycelial extracts was estimated by studying the rate of chymotrypsin catalyzed cleavage of the test peptide, and expressed as the first-order rate constant, k (s⁻¹). Higher first-order rate constant signified greater PPIase activity in the extract. The amount of total proteins used for this assay is given in the parenthesis. Bovine serum albumin (BSA) was used as a negative control. The cyclophilin- and FKBP-associated PPIase activity was estimated by the extent of inhibition in the presence of 100 nM cyclosporin A (CsA) and 2 μM FK506, respectively. Control refers to the cultures grown in Sabouraud medium without supplementing with NaCl. Changes in the total (b) and specific PPIase activity (c), and total soluble proteins (d) in the mycelia of *Penicillium oxalicum* grown in the presence or absence of salt. (e) Effect of cyclosporin A (CsA) and FK506 on mycelial PPIase activity. The values depict the mean of three biological replicates ± standard error and are significant at $P \leq 0.001$ (Tukey-HSD test; $\alpha = 0.05$). The values with distinct letters are significantly different. (DAI: days after inoculation).

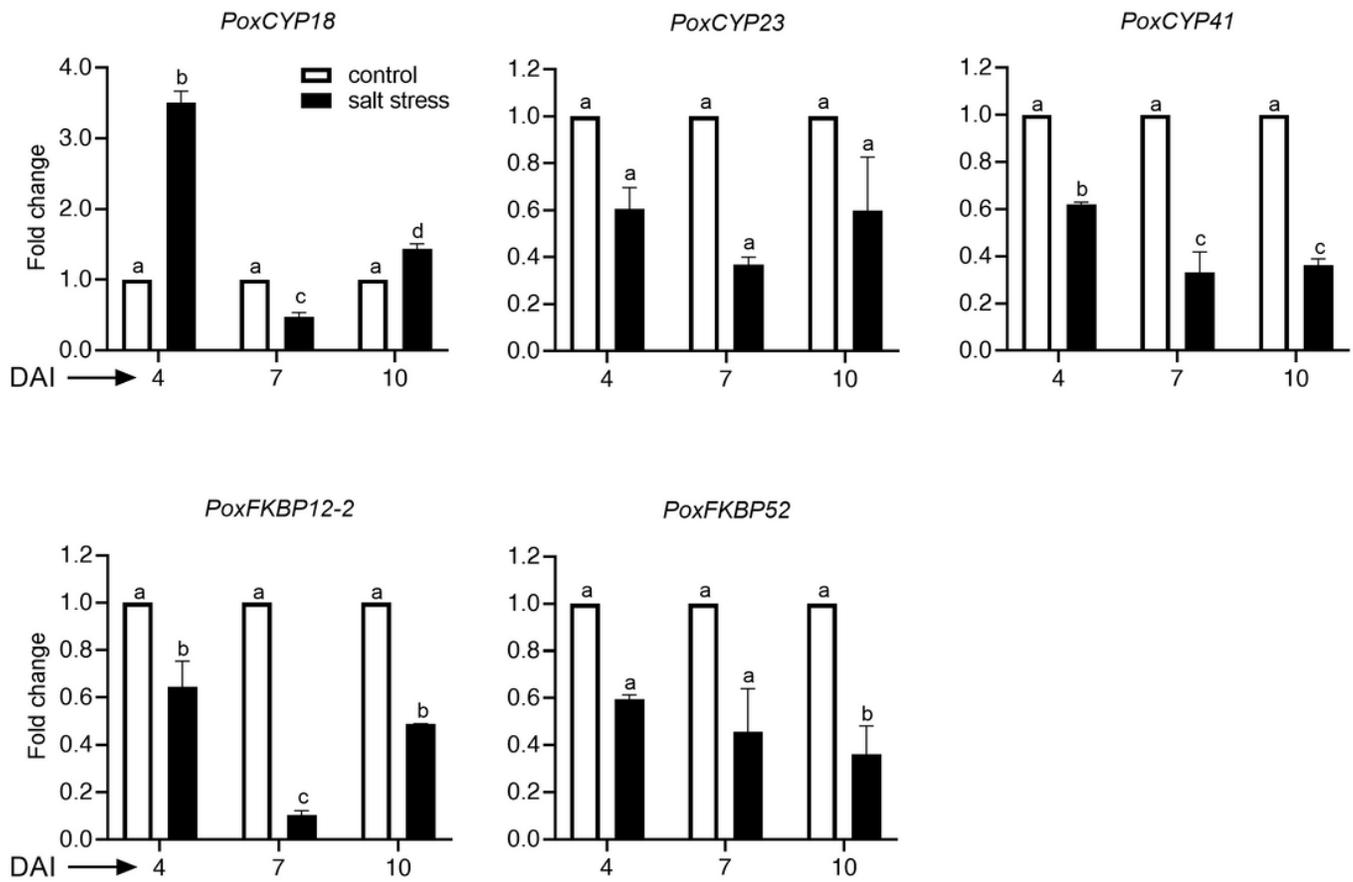


Figure 5
 Effect of salt stress (15% NaCl) on expression of cyclophilin and FKBP genes in the mycelia of *Penicillium oxalicum*. Changes in the transcript levels were estimated by Real-time PCR analysis using ACTIN gene as reference. The fold change presented is with respect to the control lacking NaCl. The values depict the mean of three biological replicates \pm standard error and are significant at $P \leq 0.01$ (Tukey-HSD test; $\alpha = 0.05$). The values with distinct letters are significantly different. (DAI: days after inoculation).

Supplementary Files

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- [Supplementarydata.pdf](#)