Cyclophilin acts as a ribosome biogenesis factor by chaperoning the ribosomal protein (PIRPS15) in filamentous fungi

Chenmi Mo 1,2, Chong Xie 2, Gaofeng Wang 2, Tian Tian 2, Juan Liu 2, Chunxiao Zhu 2, Xueqiong Xiao 1,2,* and Yannong Xiao 2,*

1 State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, People’s Republic of China and 2 Hubei Key Laboratory of Plant Pathology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, People’s Republic of China

ABSTRACT

The rapid transport of ribosomal proteins (RPs) into the nucleus and their efficient assembly into pre-ribosomal particles are prerequisites for ribosome biogenesis. Proteins that act as dedicated chaperones for RPs to maintain their stability and facilitate their assembly have not been identified in filamentous fungi. PlCYP5 is a nuclear cyclophilin in the nematophagous fungus Purpureocillium lilacinum, whose expression is up-regulated during abiotic stress and nematode egg-parasitism. Here, we found that PlCYP5 co-translationally interacted with the unassembled small ribosomal subunit protein, PIRPS15 (uS19). PIRPS15 contained an eukaryote-specific N-terminal extension that mediated the interaction with PlCYP5. PlCYP5 increased the solubility of PIRPS15 independent of its catalytic peptidyl-prolyl isomerase function and supported the integration of PIRPS15 into pre-ribosomes. Consistently, the phenotypes of the PlCYP5 loss-of-function mutant were similar to those of the PIRPS15 knockdown mutant (e.g. growth and ribosome biogenesis defects). PlCYP5 homologs in Arabidopsis thaliana, Homo sapiens, Schizosaccharomyces pombe, Sclerotinia sclerotiorum, Botrytis cinerea and Metarhizium anisopliae were identified. Notably, PlCYP5-PIRPS15 homologs from three filamentous fungi interacted with each other but not those from other species. In summary, our data disclosed a unique dedicated chaperone system for RPs by cyclophilin in filamentous fungi.

INTRODUCTION

Ribosomes are representative macromolecules that exist in all biological cells. They are essentially responsible for cellular protein synthesis (1). Ribosomes consist of two subunits: large and small. In Saccharomyces cerevisiae, the large subunit (60S) contains 25S, 5.8S and 5S rRNA and 46 ribosomal proteins (RPs), while the small subunit (40S) harbors 18S rRNA and 33 RPs (2). Ribosomes mature through a rapid and orderly assembly process to satisfy the protein requirement of biological cells. This process primarily occurs in the nucleus, where the 35S rRNA precursor (pre-rRNA) co-transcriptionally recruits specific RPs and numerous assembly factors to generate the 90S pre-processome (3–5). After progressive cleavages of pre-rRNA, the 90S dissociates into the precursors of the large (pre-60S) and small ribosomal subunits (pre-40S) (3). These two precursors are immediately transported to the cytoplasm, where they associate with additional RPs to form mature ribosome (6–8). Interference with any of these processes results in defective ribosome biogenesis (9).

Efficient nuclear transport and assembly of RPs are crucial for ribosome biogenesis. However, little is known about the mechanisms that mediate the transport of RPs from cytoplasm to nucleus. Newly synthesized RPs tend to aggregate due to their ever-present basic regions and unfolded extensions prone to nonspecific interactions (10). Accordingly, cells employ a general chaperone system, such as the nascent polypeptide-associated complex (NAC), stress 70-B/ribosome-associated complex (SSB/RAC), and importins to protect these aggregate-prone RPs (11–14). Moreover, different chaperones in S. cerevisiae associate with and solubilize specific RPs, which facilitates nuclear import of RPs and their integration into pre-ribosome, known as dedicated chaperones (15). Five chaperones interact with the RPs of 60S, including Rrb1, Acl4, Sqt1, Syo1 and Bcp1 (15–19), and three chaperones interact with
the RPs of 40S, including Yar1, Tsr2 and Tsr4 (20–23). Remarkably, the function as a dedicated RP chaperone appears to be conserved among homologs across species. For instance, a chaperone, the arginine methyltransferase 3 in Arabidopsis thaliana and Schizosaccharomyces pombe interacts with RPS2 and regulates ribosome biogenesis (24,25). A human chaperone, PDCD2 as well as its yeast homolog Tsr4, co-translationally interacts with RPS2 to facilitate its assembly (23,26). However, it is unclear whether this dedicated chaperone system is conserved in filamentous fungi.

Cyclophilins (CYPs) are ubiquitous proteins and belong to the peptidyl-prolyl cis-trans isomerases (PPIases) that exhibit catalytic activity in protein folding (27). With this feature, CYPs are involved in many biological processes across multiple species, such as cell morphogenesis, transcriptional regulation, abiotic stress resistance, and virulence (28–31). In addition, CYPs possess PPIase-independent chaperone-like activity, which prevents the aggregation of multiple proteins through direct binding (32–34). Recently, the biological function of CYPs as chaperones has been emphasized. The plant CYP40 acts as a co-chaperone of Hsp90 to promote microRNA activities by facilitating the binding of small ligands to Ago1 (35). The CYP40 homolog in Drosophila melanogaster has also been identified as the Hsp90 co-chaperone, which is essential for spermatogenesis and modulation of Ago2-RISC formation (36). Nevertheless, other possible chaperone functions of CYPs remain to be explored.

Purpureocillium lilacinum is a filamentous fungus of the phylum Ascomycota and is widely used to control plant-parasitic nematodes due to its ability to parasitize nematode eggs and the nematocidal activity of fungal metabolites (37–40). However, the molecular mechanism underlying its parasitism remains unclear, which undermines the field application of this fungal biocontrol agent. In our previous study, we identified the CYP family of P. lilacinum and found the gene expression of PlCYP5, an RNA recognition motif (RRM)-containing CYP gene, was increased upon infection of nematode eggs (41). In addition, it was also increased upon exposure to abiotic stressors (41), implying versatile roles of PlCYP5. Surprisingly, the model fungus S. cerevisiae lacks the homolog of PICYP5. Its homologs in A. thaliana and S. pombe regulate transcription by interacting with the C-terminal domain of RNA polymerase II (42,43). However, there was no such interaction in P. lilacinum. Here, we aimed to identify a novel function of the RRM-containing CYP and determine its effect on cellular ribosome biogenesis.

**MATERIALS AND METHODS**

**Strains and growth condition**

The *P. lilacinum* wildtype strain 36-1 was isolated from the egg surface of *M. incognita* and was cultured on PDA at 28°C for reproduction and on CZA at 28°C for biological phenotype determination. Gene mutation strains were cultured on potato dextrose agar (PDA) supplemented with 1.2 mg/ml G418 sulfate and gene complementary strain was cultured on PDA supplemented with 2 mg/ml glufohsinate ammonium. *Escherichia coli* strain was cultured in Luria-Bertani broth (LB) medium at 37°C.

**Sequence analysis**

The conserved protein domain of PICYP5 was identified using the PROSITE (https://prosite.expasy.org/). Sequence multiple alignment was performed using the MUSCLE program of MEGA 7.0 with default parameters, and the alignment result was applied to generate a phylogenetic tree with 1000 bootstraps. For protein structure prediction, the amino acid sequences of proteins were submitted to I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), and the most reliable result was utilized.

**Fungal transformation**

Transformation of *P. lilacinum* was performed based on the polyethylene glycol (PEG)-mediated protoplast transformation described previously with some modifications (44,45). Briefly, to produce protoplasts, 50 μl of a 1 × 10⁵ conidia/mL suspension of wildtype strains was inoculated into 100 ml tryptone-glucose medium (10 g/l tryptone, 10 g/l glucose) and shaken at 28°C. At 2 dpi (day post-inoculation), the mycelium was collected by filtering through three layers of lens paper and washing with 0.7 M NaCl to remove conidia and medium. The mycelium was then digested with 10 mg/ml lysing enzyme (Sigma, USA) and 1 mg/ml snailase (Solarbio, China) mixed solution at 120 rpm at 30°C for 4 h. The protoplasts were harvested by filtering through three layers of lens paper and washing with 0.7 M NaCl. After centrifugation at 5000 rpm at 4°C for 6 min, the protoplasts were suspended in STC solution (1.2 M sorbitol, 10 mM Tris–HCl (pH 7.5), 50 mM CaCl₂) and adjusted to the concentration of 1 × 10⁵ protoplasts/ml.

For the transformation, 100 μl of protoplasts were mixed with 2–5 μg of DNA fragments, and TEC solution (10 mM Tris–HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM CaCl₂) was added up to 160 μl. The sample was gently mixed and incubated with ice for 20 min. 160 μl of 60% PEG 3350 dissolved in 0.12 M 4-morpholinopropanesulfonic acid was added dropwise and incubated at 28°C for 30 min. After incubation, 1 ml of STC was added and mixed gently. The mixture was centrifuged at 5000 rpm at 4°C for 6 min to recover the protoplasts, which were then suspended in 250 μl STC. Each 50 μl of protoplasts was spread on a PDA plate supplemented with 10 g/l molasses, 0.6 M/l sucrose, 0.3 g/l yeast extract, 0.3 g/l tryptone and 0.3 g/l casein peptone, and incubated at 28°C. After 24 h, the plates were overlaid with T-top medium containing 1.2 mg/ml G418 sulphate, and the transformants were selected 2–7 days later.

**Subcellular localization**

All primers used in this study were listed in Supplementary Table S1. The promoter of *gpdP* gene, which was the homologous gene of Aspergillus nidulans *gpdA* in *P. lilacinum*, was amplified with the primer pairs PgpdP-F/PgpdP-R. The primer pairs, eGFP-F/eGFP-R and PICYP-F/PICYP5-R, were used to clone the enhanced green fluorescent protein (*eGFP*) and *PICYP5* gene, respectively. Overlap extension PCR was conducted to fuse these three fragments with primer pairs PgpdP-F/PICYP5-R, generating the PgpdP::eGFP::PICYP5 expression cassette. To observe the
localization of truncated PlCYP5, PlCYP5-F/PlCYP5_331- R and PlCYP5_332-478-F/PlCYP5-R were used to amplify PlCYP5_331 and PlCYP5_332-478 fragments, respectively and fused using the same strategy. PgpP:eGFP expression cassette was used as control. Transforms were screened on medium with 1.2 mg/ml G418 and further identified via PCR using the primers PgpP-J-F/PlCYP5-J-R. The blastospores and hyphae were visualized under a confocal laser scanning microscope.

Gene knockout and complementation
Approximately 1.3 kb of upstream and downstream fragments of the PlCYP5 gene was cloned with the primer pairs, PICYP5L-F/PlCYP5R-R and PICYP5R-F/PlCYP5R-R, respectively, which were named as CSL and CSR. The G418 resistance gene NPTII was split into two fragments, NP and PT, with 1.0 kb repetitive region and cloned with primer pairs, NP-F/NP-R and PT-F/PT-R. Overlap extension PCR was then conducted to generate the CSLNP and PTCSR fragments with primer pairs CSL-F/NP-R and PT-F/CSR-R, respectively. The CSLNP and PTCSR were co-transformed into wildtype protoplasts. Transforms were screened with 1.2 mg/ml G418. Gene replacement required four primer pairs for verification. CSLJ-F/NPJ-R and PTJ-F/CSRJ-R verified the upstream and downstream regions, respectively. NP-F/PT-R verified the NPTII gene, and PICYP5J-F/PlCYP5J-R verified the target gene. The same strategy was used for the knockout of the PIRPS15 gene.

Biological phenotype experiments
To assess differences in growth, aliquots of 1.5 μ1 1 × 10⁵ conidia/ml suspension of each strain were spotted on the center of CZA and PDA plates. All spotted plates were incubated at 28°C for 14 days, followed by measuring the diameter of each colony.

To assess the response of each strain to abiotic stress, the same spotted method was conducted. CZA plates were supplemented with the following chemicals: 1 M NaCl, 1 M KCl, 1.2 M sorbitol, 0.1% SDS, 0.15 mg/ml Congo red or 5 mM H₂O₂. After incubation at 28°C for 14 days, the diameter of each colony was measured.

The conidia production capacity of each strain was determined by spreading 100 μ1 of a 1 × 10⁵ conidia/ml suspension per CZA plate. After 14 days of dark culture at 28°C, three plugs (6 mm diameter) were bored from each plate using a puncher, and the conidia of the three plugs were released into 1 ml of 0.02% Tween 80 through vibration. The conidial concentration was quantified using a hemocytometer and converted to the number of conidia per cm² plate culture. The blastospore production capacity of each strain was determined by inoculating two fresh plugs into 150 ml PDB. After shaking at 180 rpm for 5 days, the culture was filtered through three layers of lens paper. The blastospore concentration was quantified using a hemocytometer and converted to the number of blastospores per ml. All experiments were conducted in triplicates.

RNA-seq and real-time quantitative PCR (RT-qPCR) analysis
For RNA-seq, 50 μ1 of 1 × 10⁵ conidia/ml suspensions of wildtype and ΔPlCYP5 were inoculated into 100 ml PDB medium respectively, followed by shaken at 28°C to collect the mycelia at vegetative growth stage. Because ΔPlCYP5 grew more slowly than wildtype, the mycelia of wildtype were collected at 2 dpi, while ΔPlCYP5 were collected at 4 dpi. The mycelia were filtered through three layers of lens paper and washed with water to remove the medium. After removing excess water, the total RNA of the samples was extracted with RNAiso Plus kit (Takara, China). After RNA quality inspection, sequencing was conducted on an Illumina MiSeq sequencing system following the manufacturer’s instructions. P. lilacinum 36-1 genome in the database (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/003/144/605/GCA_003144605_1_ASM314460v1) was used as a reference for mapping the reads using HISAT2 v2.0.1 to obtain reads count (46). The counts were normalized by HTSeq v0.6.1, and differentially expressed genes were identified using the edgeR package with the false discovery rate (FDR) < 0.05 and log₂(FoldChange) ≥ 1. The Blast2GO program was used to get Gene ontology (GO) annotation. The total RNA used for RNA-seq was also employed to analyze the expression levels of the target genes by RT-qPCR. The RNA samples were treated with the DNA-free™ DNA Removal Kit (Invitrogen™, USA) to remove DNA, and then the RNA was used to generate the first strand of cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Gene expression abundance was analyzed using the Bio-Rad CFX96 Real-Time System and SsoFastTM EvaGreen Supermix (Bio-Rad, Hercules, CA, USA). The fold change of gene expression was calculated in comparison with the control by the 2−ΔΔCt method.

Protein interaction assays
For the Y2H assay, plasmid pGBKKT7 (BD) expressing bait protein and plasmid pGADT7 (AD) expressing prey protein were co-transformed into the yeast strain Y2H GOLD (Clonetech, China). Transforms were screened by SD/-Trp-Leu and validated by PCR using general primers of BD and AD. The interactions were assessed by spotting transforms in 10-fold dilution onto SD/-Trp-Leu supplemented with X-α-galactosidase (X-α-gal) and Aureobasidin A (AbA) and SD/-Trp-Leu-His-Ade supplemented with X-α-gal and AbA, followed by incubation for 3 days at 30°C.

To analyze the interactions between PlCYP5 and PIRPS15 in vivo, Co-immunoprecipitation (Co-IP) was performed as follows. GFP-PlCYP5 and myc-PIRPS15 were co-expressed in P. lilacinum wildtype. The fresh mycelium was collected and ground into powder in liquid nitrogen. Total protein was isolated by adding RIPA lysis buffer (Beyotime, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1% proteinase inhibitor cocktail (Sigma, USA) and then centrifuged at 13 000 rpm at 4°C for 30 min to remove cell debris. The protein extract was subjected to IP assay using anti-GFP affinity sepharose (Diaan, China). After incubating at 4°C for 8 h, the sepharose
was washed three times with RIPA lysis buffer. The bound proteins were eluted by boiling for 5 min in protein loading buffer and subjected to immunoblot with c-myc antibody (Proteintech, USA). GFP co-expressed with myc-PIRPS15 in wildtype was used as control.

To validate the direct interactions between PICYP5 and PIRPS15, or between truncated PICYP5 and PIRPS15, the open reading frame of PICYP5 or its truncations was cloned into plasmid pGEX-6P-1 containing a GST tag. Then the plasmid was transformed into E. coli Rosetta (DE3). Cells were grown in 50 ml of LB medium and protein expression was induced at an OD$_{600}$ of 0.6–0.8 by the addition of isopropyl-beta-D-thiogalactoside to a final concentration of 0.2 mM. After cultured at 16°C overnight, cells were suspended in 5 ml PBS supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed ultrasonically. Recombinant GST-PICYP5 (or truncated PICYP5) was immobilized on Glutathione Sepharose (GE healthcare, USA), and incubated with E. coli, and then cross-linked to the membrane by UV. Hybridization was performed overnight at 50°C using the following labelled DNA probes (GE Healthcare): 18S (5′-CTACTACATCCAAAGGAAAGGCAGCAGCGCGCAATTACCAATCCCACAC-3′), 28S (5′-GGAGTTTTCGTGATGCGAGTTCCGG GTGTAAGACCCCTACGCCGTAAAT-3′), 5′-ETS (5′-CCACCAGTAACTTGGAAAATCTCTCCGGCG CTGAAACACCGCAGCGTTAGGACA-3′) and ITS1 (5′-CGAGTTTATAAACCATCTACCCACCTGTGTA ACCTTACCTAGTTCCTCGG-3′). The membranes were washed three times for 10 min at 50°C in wash buffer (GE Healthcare), and signals were detected using ChemiDoc Touch Imaging System (Bio-rad, USA).

**Northern blot**

Total RNA of *P. lilacinum* was prepared by the standard method using TRIzol reagent (Takara, China). The appropriate amount of total RNAs was separated on 1.2% agarose gel. RNAs were then transferred onto nylon membranes and then cross-linked to the membrane by UV. Nucleotide analysis of total RNAs was performed in triplicate. The appropriate amount of total RNAs was separated by SDS-PAGE and analyzed by Coomassie Blue staining. E. coli strains expressing PICYP5$^{1-993}$ and PIRPS15 alone were used as control.

**Statistical analysis**

The statistical significance of differences was examined using the two-way ANOVA analysis, followed by Bonferroni’s post-test. Data were presented as the mean ± standard deviation (SD) and the corresponding p-value was indicated in the figure legends.
RESULTS

PICYP5 is a nuclear RRM-containing CYP

Two characteristic sequences in PICYP5 were identified by functional domain analysis; 1–172 amino acids (aa) formed the cyclophilin-like domain (CLD) and 249–331 aa comprised the RNA recognition motif (RRM) (Figure 1A). Hence, we defined the 173–248 aa with no functional annotation as the interval region (IR), and the charged amino acid-enriched region after RRM as the charged region (CR) (Figure 1A). The CLD and RRM of PICYP5 are highly conserved among its homologous proteins, while the IR and CR are poorly conserved (Figure 1A, Supplementary Figure S1). Phylogenetic analysis showed that PICYP5 was clustered together with its homologs from the Hypocreales fungi, suggesting that they have a similar function (Figure 1B).

In the previous study, we demonstrated that PICYP5 localized in the nucleus when transiently expressed as enhanced green fluorescent protein (eGFP)::PICYP5 fusion protein in Nicotiana benthamiana (41). The nuclear localization of PICYP5 was further confirmed by expressing the full length of PICYP5 fused with eGFP in the hyphae of P. lilacinum (Figure 2B). PICYP5 harbored two nuclear localization signals (NLSs) at the C-terminal (Figure 2A). Deletion of CR containing the NLSs (PICYP51-331) prevented PICYP5 from entering the nucleus, while CR (PICYP5312-478) alone localized in the nucleus, pointing to the importance of the NLSs for PICYP5 nuclear localization (Figure 2B).

PICYP5 is involved in the growth, development, and virulence of P. lilacinum

To explore the function of PICYP5, the gene deletion (ΔPICYP5) and complementary (ΔPICYP5::PICYP5) strains were obtained through homologous recombination and verified via Southern blotting (Supplementary Figure S2). The ΔPICYP5 strain showed a significantly attenuated growth on the potato dextrose agar (PDA) and Czapek-Dox agar (CZA) plates (Figure 3A and B). When cultured on the water agar plates, ΔPICYP5 displayed thinner hyphal tips and hyperbranching compared with the wildtype and ΔPICYP5::PICYP5 (Figure 3A). The conidia and blastospore production of ΔPICYP5 was significantly decreased compared with those of the wildtype and ΔPICYP5::PICYP5 (Figure 3C and D). Moreover, the ΔPICYP5 strain had an increased sensitivity to abiotic stresses (Supplementary Figure S3).

As a nematode bio-control fungus, the nematocidal activity of wildtype and mutant strains was also investigated. To exclude the effect of growth differences between strains on fermentation, we determined the time point for each fermentation of those strains and found that the amount of proteins in ΔPICYP5 fermentation was significantly less than that of the wildtype and ΔPICYP5::PICYP5 (Figure 3G).

To determine the functional domain of PICYP5, we divided PICYP5 into four segments, including CLD, IR, RRM and CR, based on the domain analysis (Figure 1A). To ensure that each fragment localizes in the nucleus as the full length PICYP5, the CLD, IR and RRM were fused with CR containing the NLSs (Supplementary Figure S4A). Each fused fragment was expressed in ΔPICYP5 strain. Intriguingly, the growth defect caused by PICYP5 gene deletion could be rescued by none of the fused fragments (Supplementary Figure S4B), suggesting that each part of PICYP5 was indispensable for its function.

Differentially expressed genes in ΔPICYP5 were enriched in ribosome biogenesis

For an in-depth understanding of the biological function of PICYP5, transcriptome analysis of wildtype and ΔPICYP5 strains was performed. A total of 2117 differentially expressed genes were identified in ΔPICYP5 compared with the wildtype. Of these, 1404 genes were upregulated, while 713 genes were downregulated in ΔPICYP5 (Supplementary Figure S5). Gene ontology (GO) enrichment showed that the upregulated genes had functions associated with ribosome biogenesis, including rRNA metabolic process, rRNA processing, ribonucleoprotein complex biogenesis, and ribosomal small subunit biogenesis (Figure 4A). Consistent with the GO enrichment analysis, genes encoding ribosomal assembly factors, including PlNop58, PlImp10, PlUtp7 and PlEnp1, were upregulated in ΔPICYP5 (Figure 4B). These were verified by RT-qPCR (Figure 4C). The carbohydrate metabolic process was also enriched and had the largest number of genes (Figure 4A), possibly because ribosome biogenesis was a major energy consuming process (48). The downregulated genes were enriched in translation and various metabolic and biosynthetic processes (Figure 4D). Interestingly, although the genes of ribosomal assembly were upregulated, the RP genes of both 60S and 40S were significantly downregulated (Figure 4E and F), which implied that the ribosomes in mutant cells were impaired. Therefore, we presumed that PICYP5 plays a role in ribosome biogenesis.

PICYP5 physically interacted with unassembled PIRPS15

To explore the molecular mechanism in which PICYP5 plays a role, a pull-down assay coupled with LC/MS was applied to identify PICYP5-interacting proteins. A total of 79 proteins were identified, of which 54 were predicted to be localized in the nucleus or shuttled between the cytoplasm and the nucleus (Supplementary Table S2). GO enrichment analysis of these interacting candidates disclosed the possible functions of PICYP5 in protein binding, nucleic acid binding, and transcription regulation (Supplementary Figure S6). Among these candidates, several proteins were RPs of the small subunit, including RPS12 (eS12), RPS15 (uS19) and RPS19 (eS19) (Supplementary Table S2). As the transcriptome analysis suggests the role of PICYP5 in ribosome biogenesis, we further investigated its interaction with
Figure 1. PICYP5 is a conserved RRM-containing CYP. (A) Multiple sequence alignment of the CLD and RRM domains of the RRM-containing CYPs in different species. Amino acids with different colored background indicate different conservation. The secondary structures were displayed above the sequences, β indicates the β fold, and α indicates the α helix. (B) Phylogenetic analysis of PICYP5 with its homologs in different species. Sequence identity between PICYP5 and its homologs was obtained by Blastp comparison and showed behind each label. At: Arabidopsis thaliana, Bn: Brassica napus, Os: Oryza sativa, Ta: Triticum aestivum, Hs: Homo sapiens, Ce: Caenorhabditis elegans, Rs: Rhizoctonia solani, Cn: Cryptococcus neoformans, Sp: Schizosaccharomyces pombe, Bc: Botrytis cinerea, Sc: Sclerotinia sclerotiorum, Bb: Beauveria bassiana, Pl: Purpureocillium lilacinum, Ma: Metarhizium anisopliae.

these RPs. In yeast two-hybrid (Y2H) assay, we found that PICYP5 interacted with PIRPS15 (Figure 5A). The interaction was further validated by in vitro pull-down assay and in vivo co-immunoprecipitation (Figure 5B and C). In contrast, PICYP7, another nuclear CYP in P. lilacinum (41), did not interact with PIRPS15 (Figure 5A). In addition, PICYP5 did not interact with PIRPS12 and PIRPS19 (Figure 5A). This indicated that PICYP5 specifically bound to PIRPS15. To explore which parts of PICYP5 contribute to the interaction, the CLD, IR, RRM, and CR of PICYP5 were subjected to interaction assays with PIRPS15. The results of Y2H and pull-down assays showed that the CLD and IR interacted with PIRPS15, while the RRM and CR did not (Figure 5D and E).

Since the newly synthesized RPS15 is incorporated into pre-ribosome during ribosome biogenesis, we investigated whether PICYP5 binds to PIRPS15 in the pre-ribosome complex or to unassembled PIRPS15. For this purpose, 3 × flag-PlNop58, 3 × flag-PlEnp1 and 3 × flag-PIRPS15 were individually expressed in P. lilacinum and purified via flag affinity sephorose. We found that all proteins were co-purified with the PIRPS12 and another ribosomal assembly
factor, PlRio2 (Figure 5F), indicating that these three proteins from the fungus bound to pre-ribosome as their homologs in S. cerevisiae. However, PICYP5 was co-purified only with PIRPS15 but not with PlNop58 and PlEnp1 (Figure 5F). Moreover, PICYP5 was present in the non-ribosomal supernatant than in the ribosome precipitation after sucrose ultracentrifugal sedimentation of cell extracts (Figure 5G). These results demonstrated that PICYP5 was not associated with pre-ribosome but bound to unassembled PIRPS15.

**Disruption of PIRPS15 phenocopied the PICYP5 mutant**

Due to the interaction of PICYP5 with PIRPS15, we suspected that PIRPS15 phenocopies ΔPICYP5. To validate this, we attempted to knockout the PIRPS15 gene. Although we generated transformants in which expression of the PIRPS15 gene was significantly decreased compared with that of the wildtype, we failed to obtain a complete knockout strain via conidial purification, implying that PIRPS15 deletion leads to lethality. Nevertheless, we found that the expression of PICYP5 was also significantly decreased in the gene knockdown strain (ΔPIRPS15_j), and the ΔPIRPS15_j strain exhibited aberrant growth and development similar to that of the ΔPICYP5 strain (Supplementary Figure S7).

We then tested whether a high expression level of the PIRPS15 gene suppressed the growth defect phenotype of ΔPICYP5. We found that overexpression of the PIRPS15 gene under a strong promoter (ΔPICYP5::PIRPS15) partially rescued the growth defect of ΔPICYP5 (Figure 6A). The polysome profile of ΔPICYP5::PIRPS15 was similar to the wildtype (Figure 6B). ΔPICYP5 showed a reduced 40S peak and an increased 60S peak, resulting in blocking the formation of 80S and fewer polysome (Figure 6B). A similar abundance pattern of ribosomal subunits was also observed in the ΔPIRPS15_j cells (Figure 6B). These results suggested that PICYP5 and PIRPS15 were involved in the same pathway. Since polysome represents the efficiency of protein synthesis, we compared the capacity of nascent protein synthesis between the wildtype and mutants by adding a detectable methionine substitute into the methionine-free medium. As expected, reduced fluorescence signal intensity was detected in the ΔPICYP5 and ΔPIRPS15_j cells (Figure 6C and D), representing fewer nascent proteins synthesized in ΔPIRPS15_j cells. Moreover, we tested the growth of the strains at low temperature and found that the growth inhibition rates of both ΔPICYP5 and ΔPIRPS15_j at 23°C and 18°C were significantly higher than those of the wildtype, suggesting that ΔPICYP5 and ΔPIRPS15_j could not synthesize sufficient ribosomes to compensate for the reduced translational ability at low temperature (Figure 6E and F). Hence, PICYP5 played a role in ribosome...
biogenesis by interacting with PIRPS15, thereby regulating growth.

**PICYP5 and PIRPS15 were required for ribosome biogenesis**

To elucidate the mechanism underlying the regulation of ribosome biogenesis by PICYP5, we designed specific probes targeting pre-rRNA and used them to detect pre-rRNA abundance in total RNA samples extracted from the wildtype, ΔPICYP5 and ΔPIRPS15_i cells (Figure 7A). Due to the difference in growth rates among these strains, we first compared the RNA samples from the equal biomass of these strains. It showed that the abundance of mature 25S and 18S rRNAs in ΔPICYP5 were significantly less than those of the wildtype, which was caused by the reduced accumulation of 35S pre-rRNA (Figure 7B and C). To explore the specific cleavage steps affected by PICYP5 and PIRPS15, we further examined the levels of pre-rRNA processing intermediates. Compared with the level of RNAs in the wildtype, increased accumulation of 35S pre-rRNA was observed in both ΔPICYP5 and ΔPIRPS15_i, representing a delayed 35S pre-rRNA cleavage. Moreover, in the ΔPICYP5 cells, the abundance of intermediate products, 35S-A2 and 33S-A2 that were obtained by cleavage at A2 sites of 35S pre-rRNA, increased (Figure 7A, D, and E). This associated with a decreased level of 20S rRNA that was generated by the cleavage at the A1 site of 33S-A2 (Figure 7A, D and E).

The role of PICYP5 in pre-rRNA cleavage implied that PICYP5 also affected the assembly of pre-ribosome. To test this, we purified 3 × flag-PIRPS12 and its associated proteins from the nuclear extracts of the wildtype and ΔPICYP5 strains and detected the abundance of pre-ribosomal assembly factors, normalized to the level of PIRPS12. Compared with the wildtype, the 90S and pre-40S assembly factors, including PlNop58, PlMpp10, and PlEnp1, were more abundant in ΔPICYP5, suggesting an enhanced association between these assembly factors and pre-ribosomes in ΔPICYP5 (Figure 7F). Since the assembly of pre-ribosomes was a cooperative process of the binding and release of assembly factors, this enhanced association suggested that PICYP5 regulated the assembly of pre-ribosomes. In addition, the abundance of PlNob1, whose homolog in *S. cerevisiae* was responsible for the 20S rRNA cleavage after being exported from the nucleus together with the pre-40S, also increased in ΔPICYP5 (Figure 7F). Together with the reduced 20S production in ΔPICYP5 (Figure 7D), PICYP5 might have also affected the pre-40S nuclear export. Thus, PICYP5 played a role in coordinating the smooth progress of ribosome biogenesis.
Figure 4. Genes related to ribosome biogenesis were up-regulated in ΔPICYP5 strain. (A) GO enrichment in the biological process of up-regulated genes in ΔPICYP5. Red arrows indicate the biological processes related to ribosome biogenesis. (B) Gene expression of ribosome biogenesis factors in transcriptome data. (C) RT-qPCR verification of the selected ribosome biogenesis factor genes. (D) GO enrichment in the biological process of down-regulated genes in ΔPICYP5. (E) Gene expression of ribosomal proteins in transcriptome data. (F) RT-qPCR verification of selected ribosomal protein genes. The error bars indicate the SD of three replicates. * denotes $P < 0.05$.

**PICYP5 protected PIRPS15 from degradation and aggregation independently of its PPIase activity**

Because PICYP5 was not associated with the pre-ribosome, it was likely that PICYP5 acted as a molecular chaperone to support the transfer of PIRPS15 to the pre-ribosome. To test this hypothesis, we purified 3 × flag-PIEnp1 and its associated proteins from cell extracts of the wildtype and ΔPICYP5 strains. Compared with the wildtype, less PIRPS15 was co-purified in ΔPICYP5 (Figure 8A). Similarly, the protein level of PIRPS15 was reduced in the total cell extracts of ΔPICYP5 (Figure 8B), suggesting that the decreased association of PIRPS15 with pre-ribosomes was due to the decreased PIRPS15 protein level in ΔPICYP5, suggesting that PICYP5 facilitated the recruitment of PIRPS15 to the pre-ribosomes by maintaining PIRPS15 protein accumulation.

Like other RPs, PIRPS15 expressed in *E. coli* was prone to aggregation as most proteins were present in the pellet (Figure 8C, lanes 2, 5 and 8). To determine whether PICYP5 prevented the aggregation of PIRPS15, PICYP5 was co-expressed with PIRPS15 in *E. coli*. However, when the full-length sequence of PICYP5 was expressed, only a small fraction of PICYP5 was soluble (Figure 5B). Because the CR of PICYP5 was expressed with low solubility, and since it did not interact with PIRPS15 (Figure 5D and E), we chose to express the CR-lacking PICYP5 (PICYP5-M1-331). We found a noticeable increase in the amount of soluble proteins (Figure 8C, lanes 1, 4 and 7). Notably, we found that a large proportion of PIRPS15 became more soluble when PICYP5-M1-331 was co-expressed (Figure 8C, lanes 3, 6 and 9).

These results demonstrated that PICYP5 increased the solubility of PIRPS15.

The CLD of PICYP5 was a primary domain for the PICYP5-PIRPS15 interaction (Figure 5D). This prompted us to investigate whether the PPIase activity contributed to the protection of PIRPS15. We previously revealed that most amino acids required for the PPIase activity on the CLD of PICYP5 were not conserved except for the Q63 amino acid (41). Indeed, PICYP5-M1-331 exhibited weaker PPIase activity than PICYP9 which was the homolog of human CYPA (Figure 8D). We then generated a mutant variant of PICYP5 (PICYP5-M1-331), which lost its PPIase activity due to the point mutation at Q63 (Figure 8D). The co-expression test showed that PICYP5-M1-331 increased the solubility of PIRPS15 as efficiently as PICYP5-M1-331 (Figure 8E), suggesting that PPIase activity of PICYP5 is not required for its anti-aggregation function.

**PICYP5 co-translationally interacted with the N-terminal extension of PIRPS15**

To gain more insights into the PICYP5-PIRPS15 interaction, we investigated the binding site in PIRPS15. PIRPS15 belongs to the uS19 family, which includes the RPS15 family in eukaryotes and the RPS19 family in prokaryotes. Hence, we first compared amino acid sequences of these two families. The protein structure of PIRPS15 was also predicted and matched with the crystal structure of RPS19 from *Thermus thermophilus* (PDB id: 1QKF). There was a conserved core region with a similar structure for both...
Figure 5. PICYP5 directly binds to unassembled PlRPS15. (A) Y2H interaction between PICYP5 and PlRPS15. The specific interaction of PICYP5 and PlRPS15 was demonstrated by negative interactions of PICYP5 with other RPs (PlRPS12 and PlRPS19) and PICYP7 with PlRPS15. DDO: SD/-Trp-Leu. QDO/XA: SD/-Trp-Leu-His-Ade supplemented with X-gal and aureobasidin A (AbA). (B) GST pull-down assay between PICYP5 and PlRPS15. The GST tagged PICYP5 was expressed in E. coli and purified via Glutathione Sepharose. Proteins were separated by SDS-PAGE, followed by western blot analysis using GST or 6×His antibodies. The bands highlighted by black arrowheads correspond to the GST and GST-PICYP5 proteins used as baits. (C) Co-immunoprecipitation (Co-IP) analysis of PICYP5 and PlRPS15. The eGFP::PICYP5 and myc::PlRPS15 were co-expressed in P. lilacinum. The protein extract was purified via anti-GFP affinity sepharose and separated by SDS-PAGE, following by western blot analysis using GFP and c-myc antibodies. (D) Y2H interaction between truncated PICYP5 and PlRPS15. PICYP5 was divided into four truncations, including the CLD, IR, RRM, and RS based on protein domain analysis. (E) GST pull-down assay between truncated PICYP5 and PlRPS15. (F) PICYP5 is not associated with pre-ribosome. 3×flag-tagged PlNop58 and PlEnp1 were expressed and purified via anti-flag affinity sepharose, respectively. Proteins were separated by SDS-PAGE and performed coomassie blue staining or analyzed by western blot to detect the presence of PICYP5, PlRPS12 and PlRio2. (G) PlCYP5 does not bind to ribosome. Non-ribosomal and ribosome-bound proteins were separated by ultracentrifugal sedimentation. Pellet and supernatant were separated by SDS-PAGE, and analyzed by western blot analysis to detect the presence of PICYP5 and PlRPS12.

proteins (Figure 9A). Apart from the core region, PlRPS15 harbored a longer N-terminal and a shorter C-terminal extension compared with TtRPS19 (Figure 9B, Figure 9C). Sequence alignment further identified 56–127 aa of PlRPS15 as the core region, and 1–55 aa and 128–152 aa as the N-terminal and C-terminal extension (Figure 9B). Based on the alignment, we divided PlRPS15 into these three parts. Each part was used for the Y2H assay with PICYP5. The results showed that the N-terminal extension interacted with PICYP5, while both the core region and the C-terminal extension did not (Figure 9C).

We further tested different length of the N-terminal extension for interaction with PICYP5 and found a robust interaction with PICYP5 when amino acid residues 51–55 (R312N35R34G55) were present (Figure 9D). Point mutation of these amino acids to alanine (A) was introduced to investigate important residues contributing to the interaction. The single mutation of I52A, N53A or G55A did not affect the interaction. In contrast, the mutation of R51A or R54A attenuated the protein interaction, and the double-point mutations R51A/R54A caused a complete loss of the interaction (Figure 9D). To investigate the physiological significance of these amino acid residues that are required for the interaction with PICYP5, we complemented ΔPlRPS15 (PlRPS15R51A/R54A) and determined the growth phenotype at low temperatures. In agreement with the Y2H results, PlRPS15R51A/R54A could not rescue the growth retardation phenotype of ΔPlCYP5 at low temperatures (Figure 9E and F). In addition, we over-expressed the wildtype PlRPS15 and PlRPS15R51A/R54A in the wildtype and ΔPlCYP5 strains and obtained transformants with low or high expression level, respectively (Supplementary Figure S8A). Then, the polysome profiles of wildtype, ΔPlCYP5, and these overexpression strains were analyzed. In the wildtype, there was no obvious difference in polysome profile among the wildtype and strains expressing PlRPS15R51A/R54A at the low and high expression level (Supplementary Figure S8B, top row). In contrast, low and high PlRPS15 expression in ΔPlCYP5 either wildtype PlRPS15 or PlRPS15R51A/R54A produced distinct results. Low expression of wildtype PlRPS15 in ΔPlCYP5 partially restored the polysome profile. However,
Figure 6. PICYP5 and PIRPS15 mutants exhibit an impaired synthesis of ribosomes and new proteins. (A) Colony growth of wildtype, ΔPICYP5 and ΔPICYP5::PIRPS15 strains. (B) Polysome profiles of wildtype, ΔPICYP5::PIRPS15, ΔPICYP5, and ΔPIRPS15 strains. Cell extracts were prepared after cycloheximide treatment and subjected to ultracentrifugal sedimentation on 7–47% sucrose density gradients. Absorbance was recorded at 254 nm. The peaks for 40S, 60S, and 80S were indicated by red arrows. (C, D) The capacity of nascent protein synthesis in the wildtype, ΔPICYP5, and ΔPIRPS15 strains. An equal amount of blastospores of each strain was collected and subjected to the reaction using Click-iT® protein reaction system. The nascent protein level was assessed by fluorescence microscope observation (C) and by determining signal intensity using a fluorescence microplate reader (D). Scale bar = 20 μm. (E) Colony growth of wildtype, ΔPICYP5, and ΔPIRPS15 strains at normal temperature (28°C) and low temperature (23°C and 18°C). (F) Growth inhibition rate of wildtype, ΔPICYP5, and ΔPIRPS15 strains at low temperature (23°C and 18°C) compared with normal temperature (28°C). The error bars indicate the SD of three replicates. *** denotes P < 0.001.

The strain with low expression of PIRPS15R51A/R54A exhibited the abnormal 60S/40S ratio as in ΔPICYP5 (Supplementary Figure S8B, bottom row). ΔPICYP5 with high expression of wildtype PIRPS15 or PIRPS15R51A/R54A could form prominent 80S peaks, but the 40S and 80S abundance in the strain expressing PIRPS15R51A/R54A was lower than that of the strain expressing wildtype PIRPS15 (Supplementary Figure S8B, bottom row). These results suggest that PICYP5 exert its function via physical interaction with PIRPS15 for the formation and stabilization of 40S.

PICYP5 might be associated with PIRPS15 in a co-translational manner as PICYP5 bound to the eukaryote-specific N-terminus of PIRPS15 like other RP dedicated chaperones, such as Yar1 (15). If PICYP5 co-translationally interacted with PIRPS15, one would expect that PIRPS15 mRNA would be co-purified with PICYP5. To explore this hypothesis, we purified PICYP5 after translation inhibition by cycloheximide, and RNA was extracted from the purified samples. The mRNA levels of PIRPS15 as well as PIRPS3, PIRPS12 and PIRPS19 were analyzed by RT-qPCR. Prior to this, we attempted to verify the interaction between P1-Yar1 and PIRPS3, two proteins whose homologs in yeast have been reported to bind co-translationally, so that this could be used as a reference for gene enrichment. However, we did not observe the interaction between Yar1 and RPS3 of P. lilacinum in the Y2H assay (Figure 9G). Nevertheless, PICYP5 and PICYP51-331 were co-purified with PIRPS15, but not PIRPS3, PIRPS12 and PIRPS19 (Figure 9H). To obtain more evidence, we investigated the effect of increased PICYP5 on the level of PIRPS15 and found that the expression of PIRPS15 was also higher in the strain with high PICYP5 expression (Figure 9I). Moreover, high expression of PICYP5 induced a more PIRPS15 protein level in the nucleus (Figure 9J), suggesting that PICYP5 facilitated the nuclear import of PIRPS15. Collectively, we concluded that PICYP5 co-translationally interacted with the N-terminal extension of PIRPS15.
**Figure 7.** PICYP5 contributes to ribosome biogenesis. (A) Schematic diagram of 35S pre-rRNA of *P. lilacinum*. The rRNA cleavage sites and the binding sites of probes used for northern blotting are indicated. ETS: external transcribed space. ITS1 and 2: Internal transcribed space 1 and 2. (B, C) Northern blot (B) and quantitative analyses (C) of pre-rRNA and mature rRNA in wildtype, ΔPICYP5 and ΔPlRPS15 corresponding to equal biomass. The strains were cultured at 28 °C to the vegetative growth stage. Total RNAs of the same biomass of the strains were isolated, separated, and transferred to a nylon membrane. The pre-rRNA and mature rRNA were detected using the following probes: probe a for detection of 35S/35S-A0, 35S-A2 and 33S-A2 pre-rRNA, probe c for detection of 35S/35S-A0, 35S-A2, 33S-A2, and 20S pre-rRNA, probe b for detection of 18S rRNA, probe d for detection of 28S rRNA. The abundance of pre-rRNA and mature rRNA in ΔPlCYP5 and ΔPlRPS15 cells is presented relative to wildtype cells from three independent replicates. (D, E) Northern blot (D) and quantitative analyses (E) of pre-rRNA and mature rRNA in wildtype, ΔPlCYP5 and ΔPlRPS15 corresponding to the same loading amount of total RNA (7 μg). The abundance of pre-rRNA and mature rRNA in ΔPICYP5 and ΔPIRPS15j cells is presented relative to wildtype cells from three independent replicates. (F) The Enhanced association of assembly factors with pre-ribosomes in the absence of PlCYP5. 3 × flag-PlRPS12 was purified from the nuclear extracts of wildtype and ΔPICYP5 via anti-flag affinity sepharose. The purified samples were analyzed by western blot to detect the presence of PIRPS12, PlNop58, PlMpp10, PlEnp1, and PlNob1. Signals were quantified by ImageQuant TL (GE Healthcare). The error bars indicate the SD of three replicates. ns denotes $P > 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$.

**Interaction between the RRM-containing CYPs and RPS15 family was unique in the filamentous fungi**

Although the PICYP5 homolog was absent in *S. cerevisiae* and other Saccharomycotina fungi, it has been identified in other species, such as HsPPIL4 in *Homo sapiens*, AtCYP59 in *Arabidopsis thaliana*, and SpRct1 in *Schizosaccharomyces pombe*. Therefore, we analyzed the interactions between the PICYP5 and PlRPS15 homologs from these species. Not surprisingly, the interactions were absent for *H. sapiens*, *A. thaliana* and *S. pombe* homologs, but were present for the homologs from filamentous fungi, including *Sclerotinia sclerotiorum*, *Metarhizium anisopliae* and *Botrytis cinerea* (Figure 10A). To determine the reason for the distinct interactions, Y2H assay was conducted to test cross-species interactions. The results showed that HsPPIL4, AtCYP59 and SpRct1 interacted with PIRPS15 with different intensities, while PICYP5 did not interact with any RPS15 from these species. This suggested that the difference in the RPS15 sequences determined whether PICYP5 and PIRPS15 homologs interact (Figure 10B). Sequence alignment of the RPS15 family proteins revealed two variable regions. One was the N-terminal extension of amino acids 1–55, and the other was amino acids 57–64 (Supplementary Figure S9). Thus, each of these two regions of HsRPS15, AtRPS15 and SpRPS15 was replaced with the corresponding sequences of PIRPS15 (Figure 10C). Y2H assay was then conducted. Notably, HsRPS15, AtRPS15 and SpRPS15 with the replacement of the N-terminal extension (1–55 aa) from PIRPS15 interacted with HsPPIL4, AtCYP59 and SpRct1 while those with the replacement of amino acids 57–64 did not (Figure 10D). These results indicated that the difference in N-terminal sequences of RPS15 between filamentous fungi and other species is a key factor for the interaction of PICYP5-PIRPS15 homologs.
DISCUSSION

Previously, we found that *PICYP5* gene is expressed during the nematode egg parasitism and upon exposure to abiotic stressors (41). These results, together with the pleiotropic phenotype of the *PICYP5* deletion mutant, implied that *PICYP5* had an essential function for multiple biological processes. In this study, we identified *PICYP5* as a physical interaction partner of *PlRPS15* (uS19) and a factor that regulated ribosome biogenesis. Additional evidence further revealed that *PICYP5* acted as a dedicated chaperone for *PlRPS15*. First, *PICYP5* was not associated with the pre-ribosomal complex. Second, RPs easily aggregated due to positively charged amino acids in the extension. A common mechanism by which the dedicated RP chaperones protected their target from aggregation is to cover these extensions through co-translational interaction. For example, the dedicated chaperone Yar1 interacted with the N-terminus of RPS3 to maintain its solubility (20,21). Sqt1 and Rrb1 interacted with the N-terminal region of RPL10 and RPL3, respectively (15). Tsr4 bound to the N-terminal 42 amino acids of RPS2 (49). Likewise, we found that *PICYP5* (*pI = 4.67*) bound co-translationally to the N-terminal extension of *PlRPS15* (*pI = 9.80*) and exhibited an anti-aggregation function to maintain its solubility. Third, *PICYP5* had a feeble enzyme activity, which was consistent with our finding that multiple residues in *PICYP5* required for PPIase activity were not conserved (41). In addition, even without the PPIase activity, *PICYP5* still interacted with and protected *PlRPS15* from aggregation, indicating that the *PICYP5* PPIase activity was not responsible for the interaction with *PlRPS15*. Collectively, we propose a
Figure 9. PICYP5 binds to the N-terminal extension of PIRPS15. (A) Protein structure match between PIRPS15 of *Purpureocillium lilacinum* and TtRPS19 of *Thermus thermophilus*. Protein spatial structure of PIRPS15 was predicted by ITASSER server and the crystal structure of TtRPS19 was downloaded from PDB (PDB id: 1QKF). (B) Multiple sequence alignment of uS19 family (including S15 of eukaryote and S19 of bacteria). The N-terminal extension, C-terminal extension, and conserved core region were indicated above the sequences. Dm: *Drosophila melanogaster*, At: *Arabidopsis thaliana*, Hs: *Homo sapiens*, Pl: *P. lilacinum*, Sc: *Saccharomyces cerevisiae*, Sp: *Streptococcus pneumoniae*, Pm: *Pasteurella multocida*. (C) Y2H interaction between PICYP5 and truncated PIRPS15. PIRPS15 was divided into three truncations including the N-terminal (1–55), core region (56–127), and C-terminal (128–152). (D) Y2H interaction between PICYP5 and the truncated N-terminals of PIRPS15. The blue arrowheads indicate the residues mutated in PIRPS15. (E) Colony growth of wildtype, ΔPIRPS15 and ΔPIRPS15::PIRPS15ΔR51A/R54A strains at normal temperature (28°C) and low temperature (23°C and 18°C). (F) Growth inhibition rate of wildtype, ΔPIRPS15 and ΔPIRPS15::PIRPS15ΔR51A/R54A strains at low temperature (23°C and 18°C) compared with normal temperature (28°C). (G) Y2H interaction between PIYar1 and PIRPS3. (H) Relative mRNA enrichment for the indicated RP mRNAs in eGFP::PICYP5, eGFP::PICYP51–331, and eGFP::PICYP5332–478 purifications versus the control eGFP purification. (I) Relative expression levels of *PICYP5* and *PIRPS15* genes in the *PICYP5* overexpression strains versus the wildtype. Two *PICYP5* overexpression strains were obtained, with low expression level (Wildtype::PICYP5–3) and high expression level (Wildtype::PICYP5–9). RT-qPCR was used to detect the mRNA levels. (J) PICYP5 facilitates the nuclear localization of PIRPS15. Nuclear proteins were extracted from the wildtype and *PICYP5* gene overexpression strains and analyzed by western blot to detect the presence of PIRPS15 and PIRPS12. The antibody histone 3 was used to quantify the blots. The error bars indicate the SD of three replicates. *** denotes *P* < 0.001.
Figure 10. N-terminal extension of the RPS15 family contributes to the interaction with RRM-containing CYPs in filamentous fungi. (A) Y2H interaction between RRM-containing CYPs and RPS15 family proteins in different species. The RRM-containing CYPs were cloned into vector pGBK17 (BD) and RPS15 family proteins were cloned into vector pGADT7 (AD). Hs: Homo sapiens, At: Arabidopsis thaliana, Sp: Schizosaccharomyces pombe, Ss: Sclerotinia sclerotiorum, Ma: Metarhizium anisopliae, Bc: Botrytis cinerea. (B) Cross Y2H interaction between RRM-containing CYPs and RPS15 family in different species. (C) Schematic diagram of sequence replacement of the RPS15 family using the corresponding sequence of PIRPS15. (D) Y2H interaction between RRM-containing CYPs and recombinant RPS15 proteins in different species.

working model for the PICYP5–PIRPS15 interaction in ribosome biogenesis (Figure 11). In wildtype cells, PICYP5 is co-tranlationally interacted with PIRPS15 in the cytoplasm, and the complex is transported into the nucleus. The protection of PIRPS15 provided by PICYP5 allows PIRPS15 to be transferred to the ribosomal assembly site, where PIRPS15 forms the 90S with proper pre-rRNA processing and assembly factors binding/release. However, in ΔPICYP5 cells, the loss of PICYP5–PIRPS15 interaction leads to the non-specific aggregation and degradation of PIRPS15, resulting in abnormal ribosome biogenesis, which is manifested by the retarded pre-rRNA processing and 90S assembly. Hence, the ΔPICYP5 cells exhibit a ribosomal stress, which ultimately compromises normal cell growth and development.

The PICYP5 mutant exhibited various growth and development abnormalities, one of which was the hyperbranching under a slow growth rate (Figure 3A). Fungal cells possess an internal homeostatic system to maintain a certain branch density under diverse growth rates caused by external factors (e.g. changes in temperature and growth nutrient) (50,51). However, the branching homeostasis was perturbed in some gene knockout mutants, resulting in branch density changes (51). In this case, our finding suggests that PICYP5 might play a role in the branching homeostasis system. We also found that the protein content in ΔPICYP5 fermentation was reduced than that in the wildtype, and the nematocidal activity of ΔPICYP5 fermentation was significantly decreased after incubation with nematodes for 3 days (Figure 3E–G). This raised a question whether the nematocidal activity of ΔPICYP5 fermentation could reach to the level of wildtype when incubation more than 3 days. To solve this, we measured the nematocidal activity lasting to 7 days, however, it showed no significant difference from the result of 3 days. This indicated that the lack of nematocidal activity of ΔPICYP5 fermentation was due to the loss of key nematocidal proteins rather than just the reduced protein content.

Transcriptome analysis revealed that a large number of ribosome-related genes were differentially expressed in ΔPICYP5, which was consistent with a previous study that discovered a transcriptional feedback response to
ribosome biogenesis impairment (52). Surprisingly, we found the genes encoding ribosomal assembly factors were generally up-regulated in ΔPICYP5 compared with those in wildtype (Figure 3B). This was reminiscent of the phenotype found in a range of RPS (RPs of 40S) mutants (53), suggesting that ΔPICYP5 might exhibit similar gene expression signatures as those of RPS mutants. However, a common phenotype of RPS mutants was up-regulation of the RP genes (53), but we found that all RP genes were down-regulated in ΔPICYP5 (Figure 3E). It is probably because the RRM-containing CYPs, including PICYP5, play an extra role in the direct regulation of the transcription process (42,43). In addition, for the discrepancy in gene expression between the RPs and ribosomal assembly factors responding to ribosome biogenesis impairment, one explanation in yeast was that these two sets of genes were controlled by distinct transcription factors. Although homologous genes of some critical transcription factors for RPs and ribosomal assembly factors in yeast were not found in P. lilacinum, such as Rap1 and Stb3 (54,55), those transcriptional factors with identified homologous genes had the same expression patterns with their target genes. For example, the transcription factor Hmo1 was down-regulated as the Hmo1-regulated RP genes, while the Dot6 and Spt6, which drove the expression of ribosomal assembly factors, were up-regulated (54,56). This suggests that these transcription factors that regulate the expression of ribosome-related genes in yeast have a similar function in filamentous fungi.

Currently, the nuclear import mechanism of numerous RPs, including RPS15 (uS19), remains unknown. The observation that PICYP5 co-translationally interacted with RIPPS15 manifested that RIPPS15 was transported into the nucleus together with PICYP5. Under this premise, we attempted to explore the mechanism of PICYP5-RIPPS15 nuclear transport. RPs enter the nucleus with the assistance of importins. In S. cerevisiae, a number of importins that transport RPs into the nucleus have been identified, including Kap104, Kap108, Kap121 and Kap123 (19,22,57). PICYP5 has been proved to possess NLSs, and RIPPS15 was also predicted to contain an NLS at the N-terminal extension. Hence, we used Y2H assay to investigate the interactions of these importins with PICYP5 and RIPPS15 in P. lilacinum, respectively. However, the result demonstrated that none of the homologs of the yeast importins in P. lilacinum interacted with either RIPPS15 or PICYP5. A possible reason was that unidentified importins mediate nuclear import of RIPPS15 or that other mechanisms than importin assist RPs in entering the nucleus in filamentous fungi. Nonetheless, it suggested that the charged N-terminal extension of RIPPS15 needed to be covered to prevent aggregation either by PICYP5 or by as-yet-undiscovered nuclear transporter.
Overexpression of PIRPS15 in the PICYP5 mutant did not completely restore its slow-growth phenotype. It might be due to the other functions of PICYP5. Aside from the CLD, PICYP5 contained another functional domain, the RRM, which was not involved in the interaction with PIRPS15 but was well known for the function of binding RNA molecules with a wide range of specificities and affinities (58,59). For example, the homolog of PICYP5 in A. thaliana (AtCYP59) regulated the RNAPII transcription and pre-mRNA processing and had the capacity to bind RNA through its RRM (42,60). Similarly, the PICYP5 homolog, SpRct1, in S. pombe also affected the RNAPII transcription (43). Subsequently, SpRct1 was proved to function in the RNAi pathway and its RRM was required for the siRNA biogenesis (61). Our study showed that PICYP5 had the same nuclear localization with AtCYP59 and SpRct1. Moreover, the transcriptome analysis identified a large number of genes differentially expressed in the PICYP5 loss-of-function mutant compared with the wild-type. It suggested that PICYP5 might also perform a similar function in transcriptional regulation through its RRM. Therefore, overexpression of PIRPS15 did not compensate for the loss of PICYP5.

A large number of assembly factors have been identified and proven essential for ribosome biogenesis since they are recruited to pre-rRNA at specific stages to assist in the pre-rRNA processing (62–64). In contrast to assembly factors, dedicated RP chaperones are not associated with the pre-ribosomal particles. Nevertheless, biological cells lacking these RP chaperones also exhibited abnormal ribosome biogenesis, such as blocked pre-rRNA processing and pre-40S/pre-60S nuclear export (2,20,23). It was probably because the absence of a dedicated chaperone usually resulted in the instability of its target protein. Indeed, we found the reduced amount of PIRPS15 in the PICYP5 knockout mutant cells and interference with the PIRPS15 gene caused a similar cellular ribosomal stress phenotype as the PICYP5 mutant. Thus, our findings highlight the importance of dedicated chaperones for RP proteins in ribosome biogenesis.

DATA AVAILABILITY
RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE179712.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We are grateful to Dr Kenichi Tsuda at Huazhong Agricultural University, Dr Pradeep Kachroo at University of Kentucky and Dr Huiquan Liu at Northwest A&F University for insightful discussions and comments on the manuscript.

FUNDING
National Natural Science Foundation of China [31872019]; National Key Research and Development Program of China [2017YFD0200603, 2018YFD0200500]; Key Research and Development Program of Jiangxi Province [20181ACF60018]; Fundamental Research Funds for the Central Universities [2662020ZKPY012]. Funding for open access charge: National Natural Science Foundation of China [31872019]; National Key Research and Development Program of China [2017YFD0200603, 2018YFD0200500]; Key Research and Development Program of Jiangxi Province [20181ACF60018]; Fundamental Research Funds for the Central Universities [2662020ZKPY012].

Conflict of interest statement. None declared.

REFERENCES
1. Melnikov, S., Ben-Shem, A., Garreau de Loubresse, N., Jenner, L., Yusupova, G. and Yusupov, M. (2012) One core, two shells: bacterial and eukaryotic ribosomes. Nat. Struct. Mol. Biol., 19, 560–567.
2. de la Cruz, J., Karbstein, K. and Woolford, J.J. Jr (2015) Functions of ribosomal proteins in assembly of eukaryotic ribosomes in vivo. Annu. Rev. Biochem., 84, 93–129.
3. Woolford, J.J. Jr and Baserga, S.J. (2013) Ribosome biogenesis in the yeast Saccharomyces cerevisiae. Genetics, 195, 663–681.
4. Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., Schäfer, T., Kuster, B., Tschochner, H., Tollervey, D. et al. (2002) 90S pre-ribosomes include the 35S pre-rRNA, the U3 snORF, and 40S subunit processing factors but predominantly lack 60S synthesis factors. Mol. Cell, 10, 105–115.
5. Kornprobst, M., Turk, M., Kellner, N., Cheng, J., Fleming, D., Kos-Braun, I., Kos, M., Thoms, M., Berninghausen, O., Beckmann, R. et al. (2016) Architecture of the 90S pre-ribosome: A structural view on the birth of the eukaryotic ribosome. Cell, 166, 380–393.
6. Zemp, I. and Kutay, U. (2007) Nuclear export and cytoplasmic maturation of ribosomal subunits. FEBS Lett., 581, 2783–2793.
7. Hurt, E. and Beck, M. (2015) Towards understanding nuclear pore complex architecture and dynamics in the age of integrative structural analysis. Curr. Opin. Cell Biol., 34, 31–38.
8. Klinge, S. and Woolford, J.J. Jr (2019) Ribosome assembly coming into focus. Nat. Rev. Mol. Cell Biol., 20, 116–131.
9. Awad, D., Prattes, M., Koller, L., Rossler, I., Loibl, M., Perlt, M., Zisser, G., Wolinski, H., Pertschy, B. and Bergler, H. (2019) Inhibiting eukaryotic ribosome biogenesis. BMC Biol., 17, 46.
10. Pillet, B., Mitterer, V., Kressler, D. and Pertschy, B. (2017) Hold on to your friends: dedicated chaperones of ribosomal proteins. Bioessays, 39, 1–12.
11. Wang, S., Sakai, H. and Wiedmann, M. (1995) NAC covers ribosome-associated nascent chains thereby forming a protective environment for regions of nascent chains just emerging from the peptidyl transferase center. J. Cell Biol., 130, 519–528.
12. Leidig, C., Bange, G., Kopp, J., Amlacher, S., Aravind, A., Wiekles, S., Witte, G., Hurt, E., Beckmann, R. and Sinning, I. (2013) Structural characterization of a eukaryotic chaperone–the ribosome-associated complex. Nat. Struct. Mol. Biol., 20, 23–28.
13. Koplin, A., Preissler, S., Ilina, Y., Koch, M., Scior, A., Erhardt, M. and Deuerling, E. (2010) A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes. J. Cell Biol., 189, 57–68.
14. Jäkel, S., Mingot, J.M., Schwarzmaier, P., Hartmann, E. and Görlich, D. (2002) Importins fulfill a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. EMBO J., 21, 377–386.
15. Pausch, P., Singh, U., Ahmed, Y.I., Pillet, B., Murat, G., Altegoer, F., Stier, G., Thoms, M., Hurt, E., Sinning, I. et al. (2015) Co-translational capturing of nascent ribosomal proteins by their dedicated chaperones. Nat. Commun., 6, 7494.
16. Iouk, T.L., Aitchison, J.D., Maguire, S. and Wozniak, R.W. (2001) RrbiP, a yeast nuclear WD-repeat protein involved in the regulation of ribosome biosynthesis. Mol. Cell. Biol., 21, 1260–1271.
17. Pillet, B., Garcia-Gomez, J.J., Pausch, P., Falquet, L., Bange, G., de la Cruz, J. and Kressler, D. (2015) The dedicated chaperone A4c escorts
ribosomal protein Rpl4 to its nuclear pre-60S assembly site. *PLoS Genet.* **11**, e1005565.

18. Calvino,F.R., Kharde,S., Ori,A., Hendricks,A., Wild,K., Kressler,D., Bange,G., Hurt,E., Beck,M. and Sinning,I. (2015) Symportin 1 chaperones SS RNP assembly during ribosome biogenesis by occupying an essential RNA-binding site. *Nat. Commun.* **6**, 6510.

19. Ting,Y.H., Liu,T., Johnson,A.W., Shie,J.T., Chen,B.R., Kumar,S.S. and Lo,K.Y. (2017) Bcp1 is the nuclear chaperone of Rpl23 in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **292**, 585–596.

20. Koch,B., Mitterer,V., Niederhauser,J., Stanborough,T., Murat,G., Rechberger,G., Berger,H., Kressler,D. and Pertschy,B. (2012) Yar1 protects the ribosomal protein Rps3 from aggregation. *J. Biol. Chem.* **287**, 21806–21815.

21. Holzer,S., Ban,N. and Klinge,S. (2013) Crystal structure of the yeast ribosomal protein Rps3 in complex with its chaperone Yar1. *J. Cell Biol.* **425**, 4154–4160.

22. Schutz,S., Fischer,U., Altstver,M., Nerurkar,P., Pena,C., Gerber,M., Chang,Y., Caesar,S., Schubert,O.T., Schlenstedt,G. et al. (2014) A RanGTP-independent mechanism allows ribosomal protein nuclear import for ribosome assembly. *Elife* **3**, e03473.

23. Black,J.J. Musalaunamkar,S. and Johnson,A.W. (2019) Tsrl is a cytoplasmic chaperone for the ribosomal protein Rps2 in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **39**, e00094-19.

24. Perreault,A., Gascon,D., D’Amours,A., Aletta,J.M. and Bachand,F. (2009) A methyltransferase-independent function for Rmt3 in ribosomal subunit homeostasis. *J. Biol. Chem.* **284**, 15026–15031.

25. Hang,R., Wang,Z., Yang,C., Luo,L., Mo,B., Chen,X., Sun,J., Liu,C. and Cao,X. (2021) Protein arginine methyltransferase 3 fine-tunes the ribosomal subunit homeostasis. *J. Biol. Chem.* **297**, 823–832.

26. Landry-Voyer,A.M., Bergeron,D., Yague-Sanz,C., Baker,B. and Bachand,F. (2020) PDCD2 functions as an evolutionarily conserved chaperone dedicated for the 40S ribosomal protein uS5 (RPS2). *Nucleic Acids Res.* **48**, 12900–12916.

27. Göthel,S.F. and Marahiel,M.A. (1999) Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell. Mol. Life Sci.* **55**, 423–436.

28. Krzywicka,A., Beisson,J., Keller,A.M., Cohen,J., Jerka-Dziadosz,M. and Klok,C. (2001) KIN241: a gene involved in cell morphogenesis in *Paramecium tetraurelia* reveals a novel protein family of cyclophilin-like molecular chaperones in *Neurospora crassa*. *Mol. Microbiol.* **39**, 731–739.

29. Chiara,W.K., Yoshida,S., Sekino,A., Ishibashi,S., Tominaga,H. and Kurum,M. (2017) The nuclear chaperone system Sch9 facilitates HSP90-mediated RISC assembly in plants. *Appl. Microbiol. Biotechnol.* **89**, 1895–1903.

30. Chen,Y., Xiao,D., Liu,J., Xiao,Y. and Xie,B. (2015) Development of a high-efficiency gene knockout system for *Pichia chlamydosporia*. *Microbiol. Res.* **170**, 18–26.

31. Kim,D., Langmead,B. and Salzberg,S.L. (2015) HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360.

32. Shiraia,C., Takai,T., Nariai,M., Horigome,C. and Mizuta,K. (2004) Ebp2p, the yeast homolog of Epstein-Barr virus nuclear antigen 1-binding protein 2, interacts with factors of both the 60 S and the 40 S ribosomal subunit assembly. *J. Biol. Chem.* **279**, 25353–25358.

33. Kos-Braun,I.C., Jung,I. and Kos,M. (2017) Tor1 and CK2 kinases control a switch between alternative ribosome biogenesis pathways in a growth-dependent manner. *PLoS Biol.* **15**, e2002445.

34. Rossler,I., Embacher,P., Piglet,B., Murat,G., Liesinger,L., Hafner,J., Unterluggauer,J.J., Birner-Gruenberger,R., Kressler,D. and Pertschy,B. (2019) Tsrl and Napl, two novel members of the multidomain cyclophilin family in the nematophagous fungus *Paecilomyces lindenii*. *J. Cell. Mol. Biol.* **43**, 257–267.

35. Chang,Y., Caesar,S., Schubert,O.T., Schlenstedt,G. et al. (2012) A methyltransferase-independent function for Rmt3 in ribosomal subunit homeostasis. *J. Biol. Chem.* **287**, 15026–15031.

36. Landry-Voyer,A.M., Bergeron,D., Yague-Sanz,C., Baker,B. and Bachand,F. (2020) PDCD2 functions as an evolutionarily conserved chaperone dedicated for the 40S ribosomal protein uS5 (RPS2). *Nucleic Acids Res.* **48**, 12900–12916.

37. Göthel,S.F. and Marahiel,M.A. (1999) Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell. Mol. Life Sci.* **55**, 423–436.

38. Krzywicka,A., Beisson,J., Keller,A.M., Cohen,J., Jerka-Dziadosz,M. and Klok,C. (2001) KIN241: a gene involved in cell morphogenesis in *Paramecium tetraurelia* reveals a novel protein family of cyclophilin-like molecular chaperones in *Neurospora crassa*. *Mol. Microbiol.* **39**, 731–739.
and Tod6 and the histone deacetylase complex RPD3L. *EMBO J.*, **30**, 3052–3064.

57. Sydorskyy, Y., Dilworth, D.J., Yi, E.C., Goodlett, D.R., Wozniak, R.W. and Aitchison, J.D. (2003) Intersection of the Kap123p-mediated nuclear import and ribosome export pathways. *Mol. Cell. Biol.*, **23**, 2042–2054.

58. Bandziulis, R.J., Swanson, M.S. and Dreyfuss, G. (1989) RNA-binding proteins as developmental regulators. *Genes Dev.*, **3**, 431–437.

59. Birney, E., Kumar, S. and Krainer, A.R. (1993) Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.*, **21**, 5803–5816.

60. Bannikova, O., Zywicki, M., Marquez, Y., Skrahina, T., Kalyna, M. and Barta, A. (2013) Identification of RNA targets for the nuclear multidomain cyclophilin atCyp59 and their effect on PPIase activity. *Nucleic Acids Res.*, **41**, 1783–1796.

61. Chang, A.Y., Castel, S.E., Ernst, E., Kim, H.S. and Martienssen, R.A. (2017) The conserved RNA binding cyclophilin, Rct1, regulates small RNA biogenesis and splicing independent of heterochromatin assembly. *Cell Rep.*, **19**, 2477–2489.

62. Yamada, H., Horigome, C., Okada, T., Shirai, C. and Mizuta, K. (2007) Yeast Rrp14p is a nucleolar protein involved in both ribosome biogenesis and cell polarity. *RNA*, **13**, 1977–1987.

63. Segerstolpe, A., Lundkvist, P., Osheim, Y.N., Beyer, A.L. and Wieslander, L. (2008) Mrd1p binds to pre-rRNA early during transcription independent of U3 snoRNA and is required for compaction of the pre-rRNA into small subunit processomes. *Nucleic Acids Res.*, **36**, 4364–4380.

64. Shu, S. and Ye, K. (2018) Structural and functional analysis of ribosome assembly factor Efg1. *Nucleic Acids Res.*, **46**, 2096–2106.