The function of the three phosphoribosyl pyrophosphate synthetase (Prs) genes in hyphal growth and conidiation in Aspergillus nidulans

Ping Jiang,† Wen-fan Wei,† Guo-wei Zhong, Xiao-gang Zhou, Wei-ran Qiao, Reinhard Fisher and Ling Lu

Abstract
Phosphoribosyl pyrophosphate synthetase, which is encoded by the Prs gene, catalyses the reaction of ribose-5-phosphate and adenine ribonucleotide triphosphate (ATP) and has central importance in cellular metabolism. However, knowledge about how Prs family members function and contribute to total 5-phosphoribosyl-α-1-pyrophosphate (PRPP) synthetase activity is limited. In this study, we identified that the filamentous fungus Aspergillus nidulans genome contains three PRPP synthase-homologous genes (AnprsA, AnprsB and AnprsC), among which AnprsB and AnprsC but not AnprsA are auxotrophic genes. Transcriptional expression profiles revealed that the mRNA levels of AnprsA, AnprsB and AnprsC are dynamic during germination, hyphal growth and sporulation and that they all showed abundant expression during the vigorous hyphal growth time point. Inhibiting the expression of AnprsB or AnprsC in conditional strains produced more effects on the total PRPP synthetase activity than did inhibiting AnprsA, thus indicating that different AnPrs proteins are unequal in their contributions to Prs enzyme activity. In addition, the constitutive overexpression of AnprsA or AnprsC could significantly rescue the defective phenotype of the AnprsB-absent strain, suggesting that the function of AnprsB is not a specific consequence of this auxotrophic gene but instead comes from the contribution of Prs proteins to PRPP synthetase activity.

INTRODUCTION
Phosphoribosyl pyrophosphate synthetase (Prs - EC: 2.7.6.1) catalyses the reaction of ATP to form 5-phosphoribosyl-α-1-pyrophosphate (PRPP) and AMP [1–5]. It has been identified that the Prs reaction product, PRPP, is involved in many different pathways, such as the pentose phosphate pathway and the de novo and salvage purine and pyrimidine nucleotide pathways, and in the biosynthesis of nucleotide coenzymes, histidine and tryptophan [2, 6–8]. Therefore, this information indicates the central importance of Prs enzymes in cellular metabolism.

Prs genes and their products are of interest not only for their importance to our understanding of biochemistry but also for their medical significance. In Homo sapiens, there are three Prs genes (PRPS1, PRPS2 and PRPS1L1) whose superactivity is connected to gouty arthritis and whose diminished activity is associated with various neuropathies [9–13]. In addition, the missense mutations of PRPS1 result in Charcot-Marie-Tooth disease type 5 (or Rosenberg Chutorian Syndrome), Arts syndrome, PRS-I superactivity and X-linked nonsyndromic sensorineural deafness [10–12, 14–16]. In prokaryotes, Prs genes have been found and sequenced from a variety of bacteria; for example, the genome of Mycobacterium tuberculosis, which is the main pathogen of tuberculosis, contains a single Prs-encoding gene, Mt-prs (Rv1017c) [6, 17, 18]. Because PRPP is required for the cell wall biosynthetic precursor decaprenyl-1-monophosphoarabinose to maintain cell integrity, this Mt-prs gene has been used as a potential drug target. In contrast, several isoforms of Prs have been cloned and described in plants, such as Arabidopsis thaliana and Spinacia oleracea, which have five and four Prs genes, respectively, and are located in different cellular organelles [3, 19, 20]. Similar to the eukaryotic system, most fungi also possess multiple Prs genes. For instance, in the filamentous hemiascomyzecete Ashbya gossypii, PRPP synthetase encoded by four genes (Aer083cp, Agrp080cp, Agrp371cp and Agrp314cp) plays...
Aspergillus nidulans PRPP synthetase in
suggest that there existed more than one minimal functional
Moreover, genetic analysis and enzyme activity detection
suggest that there existed more than one minimal functional
unit capable of synthesizing phosphoribosyl pyrophosphate
in S. cerevisiae [20, 22–28]. These results also suggest that
Prs may have their own working paradigms in different species.

In our previous study, we found that there are three putative
annotated genes (AnprSA, AnprSB and AnprSC) encoding
PRPP synthetase in Aspergillus nidulans, according to the
Aspergillus genome database (www.aspgd.org/). Moreover, we
defined the AnPrs family as a suppressor of the
septum initiation network (SIN) and that it acts antagonistically
against SepH, a main component of SIN, so the down-
regulation of the AnPrs family can bypass the requirements
for SIN in septum formation and conidiation [29]. However,
knowledge about how AnPrs family members work
together and contribute to total PRPP synthetase activity is
limited. In this study, we used constructed deletion and conditional strains for three Anprs genes to identify that AnprsB
and AnprsC are auxothrophic genes, i.e. either deletion of
AnprsB or AnprsC confers auxothrophic requirement for the
downstream product of PRPP. In comparison, AnprSA is a
non-auxothrophic gene. Moreover, we found that these three
genes have different expression profiles at different developmental stages. The important functions of AnprsB and
AnprsC are not irreplaceable but are mainly due to the contribution of Prs proteins to PRPP synthetase activity.

METHODS

Strains, media, culture conditions, plasmids and transformation
A list of Asp. nidulans strains used in this study is provided in
supporting Table 1. The media YAG (yeast + agar + glucose
media), YUU (YAG + uridine + uracil), MMPGRU (MM +
pyridoxine + glycerol + riboflavin + uridine + uracil),
MMPDR (MM + pyridoxine + glucose + riboflavin),
MMPDRU (MM + pyridoxine + glucose + riboflavin + uridine + uracil) and MMDRU (MM + glucose + riboflavin +
uridine + uracil) and the Prs rescued medium MM (MM +
pyridoxine + glucose + riboflavin + uridine + uracil + adenine + guanine + histidine + tryptophan + AMP) as well as the
concentration of the relative components are referred from
previous references [29–35]. Growth conditions and derepression conditions for alcA(p)-driven expression were as previously described [36]. Expression of tagged genes under the control of the alcA promoter was regulated by different carbon sources: derepressing on glycerol and repression on glucose. Standard DNA transformation procedures were used for Asp. nidulans [37, 38].

Constructions of gene replacement strains
A strain containing the AnprsB-null mutation was created by
double joint PCR [39]. The Aspergillus fumigatus pyrG
gene in plasmid pXDRFP4 and the Asp. nidulans pyrA gene in plasmid pQA-pyro were used as selectable nutritional
markers for fungal transformation, respectively. The
linearized DNA fragment 1 which included a sequence of
about 686 bp that corresponded to the regions immediately
upstream of the AnprsB start codon was amplified with the
primers ΔAnprsB-p1 and ΔAnprsB-p3 (Table 2). Linearized
DNA fragment 2 including a sequence of about 541 bp that
 corresponded to the regions immediately downstream of
the AnprsB stop codon was amplified with primers
ΔAnprsB-p4 and ΔAnprsB-p6 (Table 2). Lastly, purified linearized DNA fragments 1 and 2 plus the pyrG gene or pyrA gene were mixed and used in a fusion PCR with primers
ΔAnprsB-p2 and ΔAnprsB-p5. The final fusion PCR prod-
ucts were purified and used to transform Asp. nidulans strains TN02A7. A diagnostic PCR assay was performed to
identify the deletion of the AnprsB gene by primers
ΔAnprsB-p1 and Diag-AfpyrG-3’ (Diag-AnpyroA-3’). The
AnprsA and AnprsC deletion transformants with the marker
of AnpyrG and AnpyroA were constructed by using a similar strategy and transformed to TN02A7. To further identify auxothrophic requirements of AnprsB and AnprsC, conidia from the ΔAnprsB and ΔAnprsC heterokaryotic transform-
ants selected by AnpyroA were inoculated into the selection medium MMDRU supplemented with the predicted downstream products (uracil/uridine, adenine, guanine, histidine, tryptophan and AMP). Oligonucleotides used in this study are listed in Table 2.

Tagging of AnprSA, AnprSB and AnprSC with GFP
To generate an alcA(p)-gfp-AnprsB fusion construct, a
909 bp fragment of AnprsB was amplified from TN02A7
genomic DNA with primer AnprsB-5’ (NotI site included)
and primer AnprsB-3’ (XbaI site included) (Table 2). The
909 bp amplified DNA fragment was cloned into the corre-
sponding sites of pLB01, yielding pLB-AnprsB-5’ [40]. This
plasmid was transformed into TN02A7. Homologous
recombination of this plasmid into the AnprsB locus should
result in an N-terminal GFP fusion of the entire AnprsB
gene under control of the alcA promoter and a fragment of
AnprsB under its own promoter. The transformants, which
were able to form the normal colony under the derepressing condition but could not grow under the repressing condi-
tion, was subjected to PCR analysis using a forward primer
(GFP-5’) designed to recognize the gfp sequence and a
reverse primer (prsB-3’) designed to recognize the AnprsB-
3’ sequence. We used a similar way tagging of AnprSA and
AnprSC with GFP.

Test transformants for heterokaryon formation
To detect if the primary transformants in ΔAnprsB and
ΔAnprsC are heterokaryons, conidia from the surface of
each of eight colonies (to avoid conidiophores as much as possible, as these multicinucleated structures could allow the re-growth of the heterokaryon) were removed to the
selective medium YAG and the non-selective medium YUU, respectively, using toothpicks that had been sterilized and then selected conidia were incubated at 37 °C for 48 h. After incubation, the growth phenotype of inoculated conidia on a solid plate had been examined [41]. If the deleted gene is essential or auxotrophic, then the null allele will be rescued by formation of a heterokaryon containing pyrG/geneX+ nuclei and pyrG+/geneX− nuclei in a common cytoplasm. These nuclei are segregated into individual conidia during asexual spore formation, forming a mixture of pyrG+/geneX+ and pyrG+/geneX− conidia on the surface of heterokaryon colonies. When this mixture is streaked on the YAG plate, the pyrG+/geneX− conidia cannot grow because they are pyrG− and the pyrG+/geneX− conidia cannot form colonies because they lack essential or auxotrophic geneX function. Thus, no colonies are formed on the YAG plate, the heterokaryon colonies. When this mixture is streaked on YUU, respectively, using toothpicks that had been sterilized after the cells had been fixed with 4.00 % paraformaldehyde (Polyscience, Warrington, PA) [42]. Differential interference contrast images of the cells were collected with a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany). These images were then collected and analysed using a Sensicam QE cooled digital camera system (Cooke Corporation, Germany) with the MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA), and the results were assembled in Adobe Photoshop 7.0 (Adobe, San Jose, CA).

**Western blotting analysis**

For immunodetection, conidia at 1×10⁷ conidia ml⁻¹ of alcA(p)-gfp-AnprsA, alcA(p)-gfp-AnprsB and alcA(p)-gfp-AnprsC were grown in 100 ml liquid medium of MMPGRUU and MMPDRUU, respectively, then incubated on a rotary shaker (QHZ-123B, Taihua CO.) at 220 r.p.m. at 37 °C for 18 h. Each mycelium was filtered through cheese cloth, moisture was blotted with filter paper and the mycelium was ground in liquid nitrogen. Then, 1 g of the well-ground fungi powder was placed into a small lysis tube by adding an appropriate amount of ceramic beads and then lysed with RIPA buffer containing 50 mM HEPES (pH 7.4), 137 mM KCl, 10.00 % glycerol, 1 mM EDTA, 1 µl ml⁻¹ pepstatin A, 1 µl ml⁻¹ leupeptin, 1 % Triton X-100, 0.10 % SDS, 1.00 % deoxycholate and 1 mM PMSF for 30 s on ice. The lysed solution was centrifuged at 12 000 r.p.m. for 15 min at 4 °C, and the supernatant was collected for Western blotting assay. For the quantification of the protein concentration, a BSA protein assay kit (Beyotime P0012, China) was used as a standard according to the manufacturer’s protocol. Finally, aliquots (40 µg for total protein) of cell lysates were separated on 10.00 % SDS-PAGE, and immunoblotting was performed as described previously with GFP antibody (1:1000, Roche Applied Science) and anti-mouse IgG conjugated to alkaline phosphatase (1:2000, Vector laboratories 94010, Burlingame, CA). The protein bands were stained by using an NBT/BCIP alkaline phosphatase colour development kit (Beyotime C3206, China) according to the manufacturer’s instructions [43, 44].

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**Table 1. Aspergillus nidulans strains used in this study**

<table>
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<th>Genotype</th>
<th>Source</th>
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<td>[43]</td>
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<td>[29]</td>
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RNA isolation for Northern analysis and real-time PCR

The wild-type TN02A7 strain was grown in liquid medium MMPGRUU at 37 °C for 6 h or 18 h and then transferred into solid MMPGRUU medium for 24 h. We then pulverized the mycelia of those strains to fine powder in the presence of liquid nitrogen. RNA purification and Northern blot analysis of total RNA and then transferred the sample to a nylon membrane. The blots were probed with nucleotide probes complementary to the mRNA of gpd, AnprsA, AnprsB, and AnprsC. The primer sequences of the oligonucleotide probes used for the Northern blots are listed in Table 2. The samples from the same cultivation conditions described above were treated with DNase I (TaKaRa), and reverse transcription PCR (RT-PCR) was carried out using the SuperScriptIII first-strand synthesis system (Invitrogen, 18080-051); cDNA was generated using an iScript Select cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using an ABI one-step fast thermocycler (Applied Biosystems), and the reaction products were detected with SYBR green (TaKaRa). PCR was accomplished by a 10 min

<table>
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<th>DNA sequence 5’–3’</th>
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<tr>
<td>AnprsA-p3</td>
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<td>AnprsA-p4</td>
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<tr>
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denaturation step at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. The transcript levels were calculated by the comparative \(\frac{\Delta CT}{\Delta CT}\) method and normalized against the expression of the actin gene in *Asp. nidulans*. Primer information is provided in Table 2 [45, 46].

**Assay of AnPrs activity**

Phosphoribosyl pyrophosphate synthetase activity was assayed following a modified version of as described in previous reference [47]. Briefly, conidial spores were inoculated in derepressing condition medium and then shaken at 220 r.p.m. at 37°C for 18 h. Then, the spores were transferred to repressing conditions and allowed to cultivate for 6 h. The mycelia were then resuspended in 1 ml of extraction buffer (50 mM KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) at pH 7.5, 10.00 % glycerol, 0.10 % Triton X-100, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 5 mM DTT) and disrupted by MP Fast-Prep 24 before being clarified by centrifugation (10,000 g for 15 min at 4°C). Measurements were performed in microplates by mixing 10 µl of the supernatant of the enzyme extract with 100–200 µl of measuring buffer (KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) at pH 7.5, 5 mM MgCl\(_2\), 3.75 mM R5P, 2 mM ATP, 3.75 mM phosphoenolpyruvate, 0.2 mM NADH, 1.5 U myokinase, 3 U pyruvate kinase and 1.5 U lactate dehydrogenase). The oxidation of NADH was measured by monitoring absorption at 340 nm using a Multiskan Spectrum (Thermo Electron, Waltham, MA, USA) at room temperature. The specific activity of AnPrs is expressed as µmol (Thermo Electron, Waltham, MA, USA) at room temperature.

**Constitutive overexpression of AnprsA, AnprsB and AnprsC in the background of the conditional strain alc(p)-gfp-AnprsB**

A plasmid containing the *gpdA-AnprsA-pyroA* fragment was created by double joint PCR. The *Asp. nidulans* *pyroA* gene in plasmid pQA-pyroA was used as a selectable nutritional marker for fungal transformation. The linearized DNA fragment 1 is included in a sequence of approximately 2172 bp of the constitutive overexpression promoter *gpdA* and was amplified with the primers *gpd-L* and *gpd-R* (Table 2). Linearized DNA fragment 2, which included a sequence of approximately 1801 bp of the ORF of the *Anprs* genes from the genome DNA of TN02A7, was amplified with primers G-Anprs-L and G-Anprs-R (Table 2). Finally, purified linearized DNA fragments 1 and 2 plus the *pyroA* gene were mixed and used in a fusion PCR with primers G-Anprs-p2 and G-Anprs-p5. The final fusion PCR products were purified and loaded into the commercial vector plasmid pEasy-Blunt Zero. Then, those constitutive overexpression plasmids were transformed into *Asp. nidulans* conditional strains alc(p)-gfp-AnprsB and cultivated in repressing medium. A diagnostic PCR assay was performed to identify the fusion fragment by primers *gpd*-L and *pyroA*-R. A similar strategy was used to construct the *AnprsB* and *AnprsC* constitutive overexpression strains which were then transformed to alc(p)-gfp-AnprsB [49].

**RESULTS**

Identification and phylogenetic analysis of three PRPP synthetase proteins in *Asp. nidulans*

To search for and identify the Prs homologues in *Asp. nidulans*, the protein sequence of budding yeast ScPrs1p was used as query via protein blast in NCBI. AnprsA encoded by AN6711.4 and two additional Prs homologues in *Asp. nidulans* were found. One of these was encoded by AN19654.4 and shared 30.60% protein sequence identity to AnprsA, here referred to as AnprsB. In comparison, another homologue protein encoded by AN3169.4 shares 26.90% protein sequence identity to AnprsA; we designated this protein as AnprsC. To further gain insight into the sequence information for the Anprs family, we compared these three proteins by sequence alignment. Although the length of these three proteins was different, they all shared the two conserved domains that identify them as Prs proteins. One belongs to the N-terminal domain of ribose phosphate pyrophosphokinase (Pribo-syltran-N), and the other one is the phosphoribosyltransferase (PRTases type I) domain (Fig. 1a). To further analyse the evolutionary relationship between the Anprs family and Prs homologues in *S. cerevisiae* in which the functions of Prs have been verified, we created a neighbour-joining tree based on the amino acid sequences of ScPrs1p-5p and AnprsA-C homologues. The tree-view results indicated that AnprsA is clustered together with ScPrs1p to form a group, whereas AnprsB shared the same cluster as ScPrs2p, ScPrs3p and ScPsr4p. Notably, AnprsC is most likely a putative homologue of ScPrs5p based on the phylogenetic relationship and the amino acid sequence identity (Fig. 1b). Furthermore, as shown in Fig. 1(c), the three Prs proteins in *Asp. nidulans* are conserved in all Aspergillus species.

**Individual gene knockout experiments show that AnprsB and AnprsC but not AnprsA are auxotrophic genes**

To verify the biological function for each of the Anprs proteins, the knockout strains for *AnprsA, AnprsB* and *AnprsC* genes were constructed individually using *ApypG* as the nutritional marker. PCR analysis showed that the *ApypG* nutritional marker was integrated into the genome at the original *AnprsA, AnprsB* and *AnprsC* loci (Fig. S1a, available in the online Supplementary Material). For the deletion strain of *AnprsA*, there was no detectable PCR band, indicating that the *AnprsA* whole-gene deletion mutant had been successfully constructed. Phenotypic analysis suggests that, except for the slightly slow growth rate, the deletion of *AnprsA* had no detectable effect on cellular morphology compared to that of the parental wild-type strain (Fig. 2a). For the *AnprsB* and *AnprsC* deletion strains, even when the ORFs of *AnprsB* and *AnprsC* were replaced with the nutritional marker *ApypG*, the *AnprsB* and *AnprsC* genes could still be detected, indicating the existence of a possible heterokaryon, as shown in Fig. S1(c, d). To better observe the resultant phenotype, we inoculated...
the haploid asexual spores from the transformants onto the selective solid media YAG; they were unable to germinate nor-
mally or form detectable colonies. However, when replicated
on non-selective YUU medium, the spores germinated and
formed colonies during the same culture time. According to
the standard protocol to detect essential genes as previously
described, the deletion of \textit{AnprsB} or \textit{AnprsC} resulted in trans-
formants only capable of surviving as the heterokaryon hyphal
form [41]. These heterokaryotic hyphae included two geneti-
cally distinct types of nuclei in the same cell. The genotypes of
these two types of nuclei are \textit{pyrG}/C0/ (\textit{AnprsB} + or \textit{AnprsC} +
) and \textit{pyrG} (\textit{DAnprsB} or \textit{DAnprsC}). Thus, using the previously
described heterokaryon rescue technique, as shown in Fig. 2
(b), we identified that both \textit{AnprsB} and \textit{AnprsC} may be essen-
tial genes. However, there was another alternative explanation
for this observation; \textit{DAnprsB} and \textit{DAnprsC} may confer auxo-
trophies that were insufficiently supplemented in the selection
media, or these genes may be required for function of the
\textit{AfpyrG} selectable marker since \textit{Anprs} is the metabolic step
immediately upstream of \textit{pyrG} in the metabolic pathway. To

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Bioinformatic and phylogenetic analysis of AnPrs homologues in selected \textit{Aspergillus} species compared to that in \textit{S. cerevisiae}. (a) Schematic diagram of the conserved domain in AnPrs family. Three AnPrs members all contain two conserved protein domains belonging to a Probosyltran-N domain family and PRTases-type I domain family based on a SMART protein search (http://smart.embl-heidelberg.de/). The numbers in the middle of each diagram indicate the varied length of amino acids in these three proteins. (b) The phylogenetic tree was constructed via MEGA 5.2. NCBI accession numbers of PRPP synthetase family in \textit{S. cerevisiae} are ScPrs1p (YKL181W), ScPrs2p (YER099C), ScPrs3p (YHL011C), ScPrs4p (YBL068W) and ScPrs5p (YOL061W) and phylogenetic tree representing the evolutionary relationship between AnPrs family and PRPP synthetase family in \textit{S. cerevisiae}. The tree shows that AnPrsA is close to ScPrs1p; AnPrsB is the potential orthologue to ScPrs2p, ScPrs3p and ScPrs4p; and AnPrsC is the homology to ScPrs5p. The scale bar represents genetic divergence of length 0.1 in units of amino acid substitutions per site. (c) Phylogenetic analysis of Prs homologues in selected \textit{Aspergillus} species. Amino acid sequences were aligned with a CLUSTALW multiple sequence alignment programmes and the phylogenetic tree was constructed shown via MEGA 5.2. Organism sources and NCBI accession numbers are \textit{Aspergillus oryzae} PrsA (AO090005000432), PrsB (AO090003001133) and PrsC (AO090012000798); \textit{Aspergillus clavatus} PrsA (ACLA007390), PrsB (ACLA049960) and PrsC (ACLA041080); \textit{Nassarius fischeri} PrsA (NFIA026920), PrsB (NFIA105300) and PrsC (NFIA063980); \textit{Aspergillus fumigatus} PrsA (Afu7g05670), PrsB (Afu4g07900) and PrsC (Afu3g13380); \textit{Aspergillus terreus} PrsA (ATEG06364), PrsB (ATEG03990) and PrsC (ATEG04065); \textit{Aspergillus niger} PrsA (An07g02210), PrsB (An04g05860) and PrsC (An02g09240); and \textit{Aspergillus flavus} PrsA (AFL2G00420), PrsB (AFL2G01905) and PrsC (AFL2G03660); The scale bar represents genetic divergence of length 0.1 in units of amino acid substitutions per site. Abbreviation: aa, amino acids.}
\end{figure}
To determine whether the related downstream metabolites catalysed by the Prs enzyme could rescue the growth defect phenotype in the AnpyroA-fused ΔAnprsB and ΔAnprsC heterokaryotic transformants, the predicted downstream products (uracil/uridine, adenine, guanine, histidine, tryptophan and AMP) were supplemented in the selection medium MMDRUU. As shown in Fig. 2(d), when the medium was supplemented with these downstream products, spores from the AnprsB or AnprsC deletion heterokaryon were able to form colonies in the absence of pyridoxine (Fig. 2c). We then streaked spores to isolate the single colony for which the wild-type AnprsB and AnprsC genes were absent by diagnostic PCR assay, indicating that ΔAnprsB and ΔAnprsC are purified. These data suggest that AnprsB and AnprsC are auxotrophs rather than essential genes (Fig. S3a, b). Above results indicate that deletion of AnprsB or AnprsC confers auxotrophic requirements of PRPP downstream products.
Conditional strains further confirm that *AnprsB* and *AnprsC* are auxotrophic genes

Aforementioned data suggest that *AnprsB* and *AnprsC* are auxotrophic genes; it is difficult to study their cellular function in detail by using the deletion strains. Instead, conditional strains were used. Thus, we constructed conditional strains in which the *AnprsA*, *AnprsB* and *AnprsC* genes were under the control of the derepressible/repressible inducible *alcA* promotor as regulated by the carbon source: repressed on glucose, derepressed on glycerol and induced on threonine. As illustrated in Fig. S2, the diagnostic PCR results indicated that three conditional strains were constructed as expected for homologous integration. We referred to *alc(p)-gfp-AnprsA* as ZGA01, *alc(p)-gfp-AnprsB* as WFA03 and *alc(p)-gfp-AnprsC* as WFA04. Western blot analysis showed that the molecular size of the GFP fusion protein was consistent with single-copy homologous integration at the *AnprsA*, *AnprsB* and *AnprsC* loci, respectively, in the derepressed medium as shown in Fig. 3(b). In contrast, under the repressed condition, these bands vanished completely, thus indicating the functionality and specificity of the alcohol-inducible *alcA* promoter in these three conditional strains. GFP-AnPrsA, GFP-AnPrsB and GFP-AnPrsC all exhibited the cytosol localization pattern but showed exclusion in the nuclear region (Fig. 3a). When these three conditional strains were inoculated on the derepressing medium (i.e. with glycerol as the sole carbon source) for 2.5 days at 37 °C, the colony size of these conditional strains was almost similar to that of the parental wild-type strain, indicating that the resulting GFP-AnPrsA, GFP-AnPrsB and GFP-AnPrsC fusions were functional in the derepressing media. In comparison, when they were grown on the repressing medium that contained glucose, AnPrsA conditional strain ZGA01 displayed an almost normal colony phenotype except with a slightly small size. As expected, neither conditional strain [AnPrsB (WFA03) or AnPrsC

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**Fig. 3.** Localization and phenotypic characterization of GFP-AnPrsA, GFP-AnPrsB and GFP-AnPrsC in the *alcA(p)-gfp-AnprsA*, *alcA(p)-gfp-AnprsB* and *alcA(p)-gfp-AnprsC* strains. (a) The localization pattern of AnPrsA, AnPrsB and AnPrsC under the derepressing medium MMPGRUU for 16 h; GFP-AnPrsA, GFP-AnPrsB and GFP-AnPrsC all exhibited cytosolic localization patterns but exclude the nuclear region. Bars, 10 µm. (b) Western blotting indicates that the GFP-AnPrsA, GFP-AnPrsB and GFP-AnPrsC fusion proteins were detected by the anti-GFP antibody at the predicted sizes of about 80.2, 62 and 74.1 kDa, respectively. + and – indicate depressing and repressing. (c) Colony phenotypic comparison of the parental wild-type (WJA01), *alcA(p)-gfp-AnprsA* (ZGA01), *alcA(p)-gfp-AnprsB* (WFA03) and *alcA(p)-gfp-AnprsC* (WFA04) conditional strains on the solid derepressing medium (MMPGRUU) and repressing medium (MMPDRUU) for 2.5 days, respectively. (d) Conidiospore germination phenotypic comparisons of *alcA(p)-gfp-AnprsB* and *alcA(p)-gfp-AnprsC* cultured in the repressing liquid medium MMPDRUU for 24 h respectively. Bars, 10 µm. (e) Nuclei staining phenotypic comparison of germlings in *alcA(p)-gfp-AnprsB* and *alcA(p)-gfp-AnprsC* cultured in the repressing liquid medium MMPDRUU for 24 h, respectively. The nuclei were stained with DAPI. Bars, 5 µm.
(WFA04)] could form visible colonies, thus suggesting that AnprsB and AnprsC are auxotrophic genes. This finding also suggests that, on the repressing medium, three conditional strains displayed a consistent phenotype with the deletion strains, indicating that the alcA promoter was able to be shut off efficiently under the repressed condition (Fig. 3c). To analyse the microscopic phenotypes during the loss of AnprsB or AnprsC individual function, the conidia of WFA03 and WFA04 were inoculated in the liquid repressing medium for 24 h at 37°C. WFA03 did not show any signs of isotropic growth, including only one DAPI-stained nucleus inside the cell, indicating that mitosis was arrested in the absence of AnprsB. In comparison, in WFA04, there were approximately 4–6 nuclei inside the enlarged spore, which was almost twice the size of the normal parental wild-type conidium, thus indicating that the loss of function of AnprsC was unable to stop mitosis immediately but resulted in an extended spore isotropic period (Fig. 3d, e).

Since we used pyrG as a selection marker while Anprs is upstream of pyrG in the pyrimidine biosynthesis pathway, to determine whether the AnprsB and AnprsC genes confer an auxotrophic requirement for growth rather than are essential, we inoculated these conditional strains on the repressing medium MMPDR with or without UU addition, respectively. As shown in Fig. 4, under the repressing condition, the AnPrsA conditional strain formed normal colonies similar to its parental wild-type strain. However, no detectable colonies were formed in conditional strain WFA03 or WFA04 for repressed AnprsB or AnprsC, no matter whether UU (uracil + uridine) was added or not on the repression medium MMPDR. This result indicates that the phenotype of growth inhibition in AnprsB and AnprsC conditional strains was due to turn off the gene expression of AnprsB and AnprsC but not due to the absence of the function of the pyrG selectable marker. Furthermore, to detect if the related downstream metabolites catalysed by the Prs enzyme could rescue the growth defect in AnprsB and AnprsC mutants, the predicted downstream products histidine, tryptophan, pyrimidine and AMP were added to the medium MMPDRUU. As expected, the auxotrophic phenotype of AnprsB and AnprsC could be significantly rescued to some extent. It further indicates that AnprsB and AnprsC are involved in the de novo and salvage purine and pyrimidine nucleotide pathways and in the biosynthesis of nucleotide coenzymes, histidine and tryptophan production. Phenotypic analysis demonstrates that the absence of the AnprsB or AnprsC gene product confers auxotrophy for uracil/uridine, adenine, guanine, histidine, tryptophan and/or AMP. The lack of repair with uracil/uridine alone likely indicates that at least one (or possibly all) of adenine, guanine, histidine, tryptophan or AMP is also required, i.e. repressing AnprsB or AnprsC expression results in auxotrophy for one or more of uracil/uridine, adenine, guanine, histidine, tryptophan or AMP. Above results further confirm that deletion of AnprsB or AnprsC gives rise to auxotrophy and are auxotrophic genes.

**AnprsB and AnprsC but not AnprsA are required during hyphal elongation**

According to previous reports, when conidia were cultured in liquid medium for 7h, most conidia germinated to form germlings. The 7h liquid culture time was referred to as the germination time point and the 18h liquid culture time was a vigorous hyphal growth time point. When the 18h liquid cultures were transferred to the solid culture plate for 24h, this time point was referred to as the sporulation period [50–52]. To further verify the time course of biological function for the Anprs family, we turned off three Anprs genes individually during the aforementioned different development stages. As shown in Fig. 5(a), when expression of AnprsA was halted during germination or the vigorous hyphal growth time point, germlings could be formed with a continually robust hyphal growth that was normal in the conditional strain compared to its parental wild-type strain. In comparison, the hyphal growth in repressed media from transferred germlings in conditional medium expressing AnprsB or AnprsC appeared to be almost halted. Through quantitative testing, the extended length of hyphae was a reduction of approximately 62.28 ±1.00% lower than those of the parental wild-type strain when AnprsC was turned off in the MMPDRUU medium. Moreover, under the same culture condition as those described above, the inhibited expression of AnprsB resulted in more severe hyphal extension defects than did AnprsC, with a hyphal length that was significantly decreased by 80.00±2.00% compared with the length in

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**Fig. 4.** Conditional strains further confirm that AnprsB and AnprsC are auxotrophic genes. The colony morphology of three AnPrs conditional strains and its parental wild-type grown on the repressed medium MMPDR (the glucose-containing medium: G), MMPDRUU (G+UU) or MMPDR supplemented by all of the downstream products of phosphoribosyl pyrophosphate synthetase (G+Suppl) at 37°C for 2.5 days. Abbreviations: G, glucose; UU, uracil/uridine, Suppl, adenine + guanine + histidine + tryptophan + AMP.
Similarly, we tested the inhibition of the growth of mycelia in liquid medium when the expression of the \textit{Anprs} gene family was inhibited. As shown in Fig. 5(c), repression of \textit{AnprsB} and \textit{AnprsC} remarkably inhibited the hyphal elongation during the hyphal growth stage. Through quantitative testing, the dry weight of the \textit{alc(p)}-\textit{gfp-AnprsB} and \textit{alc(p)}-\textit{gfp-AnprsC} strains was approximately 3.40- and 1.88-fold lower, respectively, than those of the parental wild-type strain on MMPDRUU (Fig. 5d). To detect the function of the \textit{AnprsA}, \textit{AnprsB} and \textit{AnprsC} genes during the sporulation period, we switched the relative conditional strains from the derepressed medium to the repressed medium during the sporulation stage. As shown in Fig. 5(e), unlike at the hyphal growth time point, inhibiting the expression of \textit{AnprsA} or \textit{AnprsB} or \textit{AnprsC} during the sporulation stage was unable to affect conidiation, suggesting that \textit{AnprsB} and \textit{AnprsC} are both required for hyphal growth but all members of \textit{AnprsA–C} are not required for sporulation.

The mRNA levels for the \textit{Anprs} family and the contribution to the total \textit{Prs} enzyme activity

Genomic information analyses indicated that all of the \textit{AnprsA}, \textit{AnprsB} and \textit{AnprsC} genes encode homologues of phosphoribosyl pyrophosphate synthetase, which implies that \textit{AnprsA}, \textit{AnprsB} and \textit{AnprsC} may cause similar defective phenotypes. However, as mentioned above, \textit{AnprsB} and \textit{AnprsC} are auxotrophic genes, but \textit{AnprsA} is not, indicating that the functions of those three genes are markedly different. To better understand the function of the three \textit{Anprs}
genes, Northern blotting and real-time PCR analysis were carried out to visualize the mRNA abundance levels of these three Anprs genes in various developmental stages. 28S and 18S RNA was the loading control for Northern blotting and actin was used as an internal reference for real-time PCR analysis, as shown in Fig. 6(a, b). As a result, at the germination time point (G 7), the AnprsA, AnprsB and AnprsC transcripts were only 10.00 %, 10.00 % and 8.00 % of the level of the relative expression of actin, respectively. In comparison, at the vigorous hyphal growth time point (V 18), the mRNA levels of AnprsA, AnprsB and AnprsC reached nearly 96.00, 27.00, and 68.00 % of the actin levels, respectively. However, the transcription of all three genes declined to the lowest level during the sporulation phase such that only 5.00 % of the AnprsB and AnprsC transcripts to the level of actin were left, compared to 12.00 % of the AnprsA in the same period. Therefore, these data indicate that the AnprsA, AnprsB and AnprsC genes have their own expression profiles at the different development stages.

These phenomena led us to consider that the contribution of different Anprs proteins may be unequal to the contribution of Prs enzyme activity. To test this hypothesis, biochemical assays for testing PRPP synthetase activity were performed according to the standard protocols described in the experimental procedures. Three Anprs conditionals and the corresponding reference strains were cultured on the repressed medium to turn off the expression during the vegetative stage. Consequently, repression of AnprsB expression in WFA03 markedly blocked the PRPP production, leaving only 49.00 ±8.00 % of the Prs enzyme activity compared to the parental wild-type. In comparison, the elimination of AnprsC expression left nearly 73.00±2.00 % of the Prs enzyme activity compared that in parental wild-type strain. However, when the AnprsA conditional strain ZGA01 was cultured under the same repressing condition, it was able to keep almost 86.00±1.00 % of the Prs enzyme activity (Fig. 6c). Taken together, these results suggest that PRPP synthetase activity was predominately dependent on the transcription level of AnprsB, followed by AnprsC and then AnprsA.

**Functional complementation test in the AnPrs family**

According to the above results, AnprsB and AnprsC probably have major roles in the AnPrs family for their contribution to Prs enzyme activity. Therefore, the deletion of AnprsB or AnprsC causes the growth inhibition, indicating that normally expressed AnprsA, AnprsC or AnprsA, AnprsB could not rescue the deleted function of AnprsB or AnprsC, respectively. To gain further insight into whether AnprsB is irreplaceable, we carried out the functional complementation test to examine the constitutive overexpression of AnprsA and AnprsC in the background of the repressed AnprsB strain. A full-length ORF sequence of AnprsA or AnprsB or AnprsC under the constitutive control of the gpdA promoter was separately

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**Fig. 6.** The mRNA levels for the Anprs family and the contribution to the total Prs enzyme activity. (a) Expression levels of AnprsA, AnprsB and AnprsC mRNA were shown by Northern blot analysis with RNA extracted from the wild-type strain throughout three developmental stages. The capital letters, G 7, V 18 and S 24, indicate the germination time point (G 7), vigorous hyphal growth time point (V 18) and sporulation period (S 24), respectively. Equal loading of RNA samples was evaluated by 28S and 18S RNA bands (l c: loading control). Numbers below the blots denoted the relative density of each band normalized as calculated using ImageJ (b v: band volume). (b) The relative mRNA levels of AnprsA, AnprsB and AnprsC, respectively, using a real-time RT-PCR assay in a wild-type strain cultured in liquid MMPGRUU medium at 220 r.p.m. for three developmental stages. The measured quantity of mRNA in each of the treated samples was normalized using 18S RNA bands (l c: loading control) as internal reference (AN3696.4) (c) The fold changes of relative Anprs enzyme activities of alc(p)-gfp-AnprsA (ZGA01), alc(p)-gfp-AnprsB (WFA03) and alc(p)-gfp-AnprsC (WFA04) after being switched to the repressing liquid medium for 6 h during the vegetative growth stage (V 18) compared to the parental wild-type. The measurements of Anprs enzyme activity in the three mutant strains were normalized to a percentage compared to wild-type strain TN02A7. Data were the means ± 50 of three sets of experiments.
transformed into the alc(p)-gfp-AnprsB strain on the repressed medium (Fig. 7a, b), resulting in three constitutive overexpression [OE means gpdA(p)::Anprs] strains that we named WFA05 [alc(p)-gfp-AnprsB::OE::prsA], WFA06 [alc(p)-gfp-AnprsB::OE::prsB] and WFA07 [alc(p)-gfp-AnprsB::OE::prsC]. As shown in Fig. S3(c), the diagnostic PCR results indicated that three constitutive overexpression strains were constructed successfully. To further ensure that the expression levels of AnprsA, AnprsB and AnprsC in these OE strains were truly overexpressed, quantitative RT-PCR analysis was determined as shown in Fig. 7(c). As expected, the mRNA level of AnprsA, AnprsB or AnprsC in these OE strains was approximately 11.25-, 50.97- or 13.94-fold higher than that in the parental wild-type strain, respectively. As shown in Fig. 7(b), colonies overexpressing AnprsB as a gene complementary test showed a similar colony phenotype to the parental strain in regard to colony size and conidiation, indicating that this system, under the control of the gpdA promoter, is functional for AnPrsB. Notably, the other OE strains were also able to restore significantly defective hyphal growth to the normal level in the alc(p)-gfp-AnprsB conditional strain under the repressed condition. The constitutive overexpression of AnprsA could partly rescue the alc(p)-gfp-AnprsB defects in the repressed condition with a slightly smaller colony size than the wild-type. In contrast, the constitutive overexpression of AnprsC could restore hyphal growth to the wild-type level with a similar colony size to that in the parental strain. However, OE::AnprsC was unable to completely rescue the conidiation defect in the repressed AnprsB background strain. To further determine whether the above colony rescue phenomena were related to the increased Prs enzyme activity induced by the constitutive overexpression of the AnprsA gene, we measured the PRS enzyme activity as shown in Fig. 7(d), the total PRPP synthetase activity of OE::prsB and OE::prsC reached almost 126.00±1.00 % and 97.00±1.00 %, respectively. However, the alc(p)-gfp-AnprsB::OE::prsA strain reached only 45.11±2.00 % of the total PRPP synthetase activity compared to its parental wild-type strain. These data demonstrate that although AnprsB is an auxotrophic gene, it is not uniquely irreplaceable because the constitutive overexpression of the non-auxotrophic gene AnprsA or the auxotrophic gene AnprsC can significantly rescue the growth defect in the alc(p)-gfp-AnprsB conditional mutant under the repressed condition.
DISCUSSION

Phosphoribosyl pyrophosphate (PRPP) is an important central compound for cellular metabolism. The formation of PRPP is catalysed by the enzyme PRPP synthetase, which is encoded by Prs genes. Prs genes exist in a variety of organisms to provide unique functions. As indicated by using comparative genomic analyses of S. cerevisiae PRS1 via BLAST search, we identified three homologous genes AnprsA, AnprsB and AnprsC that encode PRPP synthetases in Asp. nidulans. The genome-scale homologue survey revealed that Anprs genes are ubiquitous and conserved among selective Aspergillus species, such as Asp. fumigatus, Aspergillus niger and Asp. oryzae. Based on the full-length alignment, AnPrsA in Asp. nidulans is most closely related to its counterpart in Asp. fumigatus, with 92.45 % identity, and is least homologous with its counterpart from Aspergillus terreus, with 90.67 % identity (Fig. S4). Comparatively, AnPrsC exhibited the highest identity with Aspergillus flavus (95.41 %) and the lowest identity (94.13 %) with Aspergillus clavatus (Fig. S5). Most notably, AnPrsB is the most conserved member among Prs members in the selected species in this study, with identities ranging from 98.75 to 99.06 %, as shown in Fig. S6. Moreover, according to the neighbour-joining evolutionary tree analysis, AnPrsA is a putative homologue of yeast ScPrs1p (identity 70.35 %), whereas AnPrsC is most likely a homologue of yeast ScPrs5p (identity 69.88 %). This reduction in identity highlights the existence of non-homologous regions in ScPrs1p and ScPrs5p [20]. Most interestingly, AnPrsB is closely related to that of ScPrs2p, ScPrs3p and ScPrs4p in yeast, with identities of 89.10 %, 88.75 and 80.74 %, respectively, suggesting that AnPrsB is likely to be an orthologue of ScPrs2p, ScPrs3p and ScPrs4p (Fig. S7). Previous studies have indicated that, in H. sapiens, the missense mutation of HsPRS1 (PRPS1) might be related to many syndromes, indicating that the function of PRPS1 plays an important role in these physiological processes [10–12, 14–16]. As shown in Fig. S8, we found that the AnPrsB protein shares 67.00 % identity with the human disease related protein HsPRS1 (PRPS1) but not HsPRS2 (PRPS2) or HsPRS3 (PRPS1L1). Most interestingly, all sites in PRPS1 with mutations related to human diseases are conserved in AnPrsB (Fig. S8). Therefore, this information suggests that studies on the function of Prs homologues in a model organism such as Asp. nidulans could provide valuable clinical indications and hints for mammalian partners related to genetics and biochemistry.

It has been reported that no single ScPRS gene is essential in S. cerevisiae but that combined deletions of double (such as ScPRS1 and ScPRS5 or ScPRS3 and ScPRS5) or triple member deletions (such as ScPRS1, ScPRS2 and ScPRS4) in five ScPRS polypeptides could cause a lethal phenotype [53]. This suggests that the five ScPRS genes might combine into certain multimeric complexes that are required for PRPP synthetase function. Interestingly, in this study, we found that AnprsA is a non-auxotrophic gene but that AnprsB and AnprsC are auxotrophic genes, i.e. the deletion of either AnprsB or AnprsC causes an auxotropic phenotype, and by using conditional strains, we further found that repressing AnprsB or AnprsC expression results in auxotrophy for more of uracil/uridine, adenine, guanine, histidine, tryptophan or AMP. This phenomenon raises a question of whether the abundant expression of the AnprsB and AnprsC genes but not AnprsA is required during all development stages. However, as shown in Fig. 6a, our findings indicate that AnprsA has an even higher relative expression level than that of AnprsB and AnprsC at the vigorous hyphal growth time point (V 18). It is possible that the individual function of AnPrsA is unable to support the activity of PRPP synthetase. Instead, the other two Anprs genes provide the major PRPP synthetase activity. In addition, our Northern blotting data suggest that the AnprsA, AnprsB and AnprsC genes have their own expression profiles at different developmental stages, indicating that they may cooperate with each other for PRPP synthetase activity. Previous studies have indicated that Ash. gossypii, a filamentous hemiascomycete, possesses four nonessential Agprs genes (Aer083cp, AGL080cp, Agr371cp and Adr314cp) and that these four genes form two homologous heterodimers, AGR371C (Agr371cp-Adr314cp) and AGL080C (Aer083cp-Agl080cp). Compared to AGR371C, AGL080C makes a major contribution to total Prs activity; the double deletion of Aer083cp and AGL080cp resulted in a large impairment in PRPP synthetase activity accompanied by growth alteration. These data suggest that different Prs members may contribute unequally to PRPP synthetase activity supported by studies of S. cerevisiae PRS gene and their products [54]. In comparison, our data demonstrate that PRPP synthetase activity is predominantly dependent on the transcription level of AnprsB, followed by AnprsA and then AnprsC (Fig. 6b, c). Therefore, loss of AnprsB or AnprsC is unable to allow the PRPP synthetase activity to reach the minimal level required for the growth in the filamentous fungus Asp. nidulans. There are two potential explanations for this phenomenon. First, the loss of AnprsB or AnprsC but not of AnprsA function could remarkably decrease the entire Prs enzyme activity in conditional strains, further indicating that AnPrsB and AnPrsC have major roles compared to AnPrsA (Fig. 6c). Second, in addition to being major contributors to the total PRPP synthetase activity, AnprsB or AnprsC may have multiple auxotrophic roles during morphogenesis. For example, some studies have reported that the constitutive overexpression of prs genes could significantly enhance the production of riboflavin in Ash. gossypii, indicating that Prs proteins may have other functions required for growth and metabolism aside from the function of total PRPP synthetase activity. In contrast, our data indicate that AnprsB is not irreplaceable, even though it is an important auxotrophic gene. Most notably, our results found that the constitutive overexpression (OE) of the AnprsA or AnprsC gene could rescue the defect of absent AnprsB in the conditional strain under the repressed condition. To further determine why overexpressing the other two Prs members could rescue the auxotrophic gene function of AnprsB, we detected the total AnPrs enzyme activity in these OE strains. As a result, OE
strains of AnprsA and AnprsC showed an almost similar level to that of the parental wild-type strain in terms of the total PRPP synthetase activity (Fig. 7). These findings clearly show that the auxotrophic function of AnprsB and AnprsC is not specific for these genes but is mainly due to the contribution of Prs proteins to PRPP synthetase activity.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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