SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

May 2013 Vol.56 No.5: 467–475
doi: 10.1007/s11427-013-4480-6

pyrG is required for maintaining stable cellular uracil level and normal sporulation pattern under excess uracil stress in *Aspergillus nidulans*

SUN XianYun[1], ZHU JuFen[1], BAO Li[1], HU ChengCheng[1], JIN Cheng[1], HARRIS Steven D.[2], LIU HongWei[1] & LI ShaoJie[1*]

[1]*State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China;*
[2]*Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0660, USA*

Received February 25, 2013; accepted March 28, 2013

Tight control of the intracellular uracil level is believed to be important to reduce the occurrence of uracil incorporation into DNA. The *pyrG* gene of *Aspergillus nidulans* encodes orotidine 5′-phosphate decarboxylase, which catalyzes the conversion of orotidine monophosphate (OMP) to uridine monophosphate (UMP). In this study, we found that *pyrG* is critical for maintaining uracil at a low concentration in *A. nidulans* cells in the presence of exogenous uracil. Excess uracil and its derivatives had a stronger inhibitory effect on the growth of the *pyrG89* mutant with defective OMP decarboxylase activity than on the growth of wild type, and induced sexual development in the *pyrG89* mutant but not in wild type. Analysis of transcriptomic responses to excess uracil by digital gene expression profiling (DGE) revealed that genes related to sexual development were transcriptionally activated in the *pyrG89* mutant but not in wild type. Quantitative analysis by HPLC showed that the cellular uracil level was 6.5 times higher in the *pyrG89* mutant than in wild type in the presence of exogenous uracil. This study not only provides new information on uracil recycling and adaptation to excess uracil but also reveals the potential effects of OMP decarboxylase on fungal growth and development.

orotidine 5′-phosphate decarboxylase, stress, uracil, sexual development

Citation: Sun X Y, Zhu J F, Bao L, et al. *pyrG* is required for maintaining stable cellular uracil level and normal sporulation pattern under excess uracil stress in *Aspergillus nidulans*. Sci China Life Sci, 2013, 56: 467−475, doi: 10.1007/s11427-013-4480-6

Orotidine 5′-phosphate decarboxylase (OMP decarboxylase; EC 4.1.1.23) is essential for the *de novo* biosynthesis of pyrimidine. It catalyzes the conversion of orotidine monophosphate (OMP) to uridine monophosphate (UMP) by decarboxylation. OMP decarboxylase is encoded by *URA3* in *Saccharomyces cerevisiae* [1], *pyrG* in *Aspergillus nidulans* [2], and *pyr-4* in *Neurospora crassa* [3]. Auxotrophic mutants with impaired OMP decarboxylase can grow normally if exogenous uridine and uracil are added to the growth medium, and their deficiency is easily complemented by genes encoding functional OMP decarboxylases. Therefore, genetic transformation systems based upon complementation of OMP decarboxylase mutants are widely used for many fungal species, such as *S. cerevisiae* [4], *A. nidulans* [5], *Penicillium chrysogenum* [6], *Aspergillus niger* [7], *Trichoderma reesei* [8] and *Aspergillus fumigatus* [9]. In filamentous fungi, the *N. crassa pyr-4* gene is a widely used selectable marker to complement OMP decarboxylase mutants [5–11].

When the phenotype of a newly generated mutant is characterized, the corresponding OMP decarboxylase-deficient parent strain is often used as a control. In this case,

*Corresponding author (email: shaojieli@gmail.com)

uracil must be added to the medium to allow the parent strain to grow. However, it is not known whether the exogenous uracil has different effects on the growth or development between *pyrG* mutants and wild type. Several lines of evidence suggest that the cellular uracil level is tightly controlled to prevent the accumulation of excess cellular uracil in fungi. In response to exogenous uracil, the transcriptional level of *FUR4*, which encodes a uracil permease for uracil transport in *S. cerevisiae* [12], is downregulated [13,14]. Similarly, exogenous uracil triggers the transcriptional down-regulation of *A. nidulans furD*, a gene encoding Fur4p-like uracil permease [15]. At the posttranslational level, the presence of exogenous uracil accelerates the degradation of Fur4p by enhancing its ubiquitylation [14]. Tight control of the cellular uracil level is believed to be important to maintain a low ratio of uracil/thymine to prevent the incorporation of uracil into DNA [15]. Replacement of thymine with uracil in regulatory regions of genomic DNA can disrupt the specificity of protein-DNA interactions [16].

In this study, we found that the loss of OMP decarboxylase in *A. nidulans* increased its sensitivity to excess uracil and its derivatives. Excess uracil and its derivatives induced sexual development in OMP decarboxylase mutants but not wild type. Transcriptomic profiling of uracil responses by DGE also revealed that genes involved in sexual development were activated by excess uracil in the OMP decarboxylase mutant.

1 Materials and methods

1.1 Strains and media

In this study, the *A. nidulans* strain FGSC A773 (*pyrG89*; *wA3*; *pyroA4*; *veA1*) was used as the parental strain. A PCR fragment containing the *N. crassa pyr-4* sequence was transformed into FGSC A773, yielding strain AHC01 (*pyrG89*; *wA3*; *pyroA4*; *pyr-4*; *veA1*). Fungal transformation was conducted as described by Miller et al. [17] and Calvo et al. [18]. ALS05 (*wA3*; *pyroA4*; *veA1*) was generated by crossing FGSC A773 (*pyrG89*; *wA3*; *pyroA4*; *veA1*) with FGSC A4 (*veA*+).

MMV medium (1% glucose, nitrate salts, trace elements, and vitamins, pH 6.5) was used for growing *A. nidulans*. The composition of trace elements, vitamins, and nitrate salts in the medium were as described by Käfer [19]. Uridine (5 mmol L^{-1} as 1×) and uracil (10 mmol L^{-1} as 1×) were added as needed. Media were solidified with 1.5% agar.

1.2 Analysis of cellular uracil and other intermediates

The spores of *A. nidulans* A773 or ALS05 were inoculated into a 300-mL flask containing 150 mL medium (MMV+1× uridine+4×uracil) and incubated at 28°C with constant agitation at 180 r min^{-1} for 30 h. Subsequently, metabolites were extracted following the method described by Ruijiter and Visser [20]. The extracted mycelia were collected and lyophilized to determine dry weight.

The extracts were filtered through 0.45-μm filters and intermediary metabolites were identified and quantified by high performance liquid chromatography (HPLC) using a COSMOSIL C18 column (4.6 I.D.×250 mm) with 5% acetonitrile and 0.04% trifluoroacetate solution as the eluent.

1.3 Studies on sexual development

To compare sexual development of the *pyrG89* mutant with that of wild type, the production of Hülle cells was evaluated. Four plugs (0.5 cm in diameter) of fungi growing on the surface of the agar medium (MMV+1×uridine+4×uracil) were harvested with a borer and homogenized in 2 mL distilled water. Hülle cells were counted with a hemacytometer under a light microscope. The average number of Hülle cells per square millimeter from three replicates was calculated.

1.4 Analysis of transcriptomic responses to excess uracil by digital gene expression profiling analysis

To compare the differences in transcriptomic responses to excess uracil between wild type and the *pyrG89* mutant, we examined genome-wide transcriptional responses to excess uracil by digital gene expression (DGE) profiling [21]. Briefly, the spores of *A. nidulans* wild type or the *pyrG89* mutant were inoculated into 150-ml flasks containing 75 mL liquid medium (MMV+1×uridine+4×uracil and MMV+1× uridine+1×uracil) and incubated at 28°C with shaking at 180 r min^{-1} for 30 h. Then RNA was extracted from three replicate samples for the DGE profiling assay. Isolation of mRNA from total RNA samples and preparation of sequencing tags were conducted using the DGE Tag Profile Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Details of the method were described by Sun et al. [22].

After treatment of raw data, all clean tags were mapped to the reference sequences (http://www.broadinsti tute.org/annotation/genome/aspergillus_group/Blast.html). To monitor mapping events on both strands, both sense and complementary antisense sequences were included in the mapping process. The clean tag numbers corresponding to each gene were counted.

1.5 Identification of differentially transcribed genes

To identify genes differentially transcribed between two samples, the number of raw clean tags in each sample was normalized to tags per million (TPM). Detection of differentially transcribed genes or tags across samples was performed according to the method previously reported, i.e., transcriptional ratios between two samples of greater than 2

and less than 0.5 were the thresholds to define differentially transcribed genes [23]. Correlations between count numbers in two parallel libraries were assessed statistically by calculating Pearson correlation coefficients. In addition to the *P*-value, the false discovery rate (FDR) was manipulated to determine differentially transcribed genes [24]. In this study, $P \leqslant 0.01$, FDR$\leqslant$0.1, and the absolute value of log2 ratio$\geqslant$1 were used as the thresholds to assess the significance of differences in gene transcription.

1.6 Biological function annotation and enrichment analysis

To reveal the biological functions of differentially transcribed genes in response to excess uracil, annotation of the important subsets of genes was performed using BLAST (ver. 2.2.23+) software against the FunCat database (http://mips.helmholtz-muenchen.de/proj/funcatDB/), with an *E*-value cut-off of 1×10^{-10}. To further highlight significant biological changes, gene set enrichment analysis was conducted with R statistical software (http://www.r-projects.org/), and the hypergeometric test algorithm was applied with the threshold values of $P\leqslant0.01$ and FDR$\leqslant$0.1.

1.7 Quantitative real-time PCR analysis

cDNA was synthesized from total cellular RNA using a cDNA Synthesis Kit (Fermentas, Burlington, Canada). PCR was performed in using a iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR-Green detection (SYBR PrimeScript RT-PCR Kit, TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China), according to the manufacturer's instructions. Each cDNA sample was analyzed in triplicate, and the average threshold cycle was calculated. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [25]. The results were normalized to level of β-tubulin transcripts. The primer pairs used for qRT-PCRs are shown in Table 1.

Table 1 Gene-specific primer pairs used for quantitative real-time PCR

Gene	Sequences of primers (5′→3′)
nsdD	QnsdDF: GGCGGAACCATCACGTTCAAGTTT QnsdDR: TAACAGTGCTTGCACTTTGCGTCG
nosA	QnosAF: ACCCATACGTTCTTCCTCCGTTCA QnosAR1: TTTCGATCGGTAGGTGTGGGCAAA
noxA	QnoxAF: ACCGAACTGAAGAGCAGGACCAAT QnoxAR: ACCAATCTCTGTCCTGGAATGCGT
fhbA	QfhbAF: AAACAACGTACTTCCTGACGGCTC QfhbAR: TCTGCTTGTCGCCAAGGAAGAGAT
glpV	QglpVF: GATCCGCCTTCAGTTTGCCATTGT QglpVR: AATCGCCCGAAGTCTCAGAGAGGTAT
upt	QuptF: AAGCCATCGACGTGCTGAAAGAGA QuptR: CTTAACCGCGGAAACCGTTGCATT
hsp20	Qhsp20F: TCTGAACATCAAGGGCCACAGTGA Qhsp20R: TAGGAAAGTTGAACGAGCGTCGGA
β-tub	QbtubF: TTCGGACGAGACATTCTGCTTGGA QbtubR: TGACAGCAGACACCAGATGGTTGA

2 Results

2.1 *pyrG89* mutation increases sensitivity to excess uracil

In fungi, the cellular uracil level is tightly controlled to prevent the accumulation of excess cellular uracil. *A. nidulans* strains lacking PyrG (orotidine 5′-phosphate decarboxylase) activity can grow only when exogenous uracil and uridine are added to the medium. The *pyrG*-defective mutants should be more tolerant than wild type to excess exogenous uracil and uridine. To test this possibility, we compared the growth of the wild-type control strain ALS05 with that of the *pyrG*-defective mutant A773 (*pyrG89*) on plates with different concentrations of uracil and uridine. Opposite to our expectations, the *pyrG89* mutant was more sensitive than wild type to excess uracil and uridine. As shown in Figure 1, after 5 d of growth, the colony sizes of the wild-type control strain on plates containing a high concentration of uracil and uridine (four times normal levels, designated as 4×uracil or 4×uridine) were similar to those on plates with normal concentrations of uracil and uridine (1×uracil, 1×uridine). In contrast, the average colony size of the *pyrG89* mutant on the 4×uracil and 4×uridine plates was 69%±2% of that on 1×uracil and 1×uridine plates. We then found that excess uracil could inhibit colony growth, and excess uridine (20 mmol L^{-1}) did not inhibit colony growth in either wild type or the *pyrG89* mutant (Figure 1). Transformation of *N. crassa pyr-4* into the *pyrG89* mutant completely rescued the uracil-hypersensitive phenotype caused by the *pyrG89* mutation (Figure 1).

On 1×UU (5 mmol L^{-1} uridine and 10 mmol L^{-1} uracil) plates, both wild type and the *pyrG89* mutant reproduced mainly by asexual sporulation (Figures 1 and 2). The colonies of both wild type and *pyrG* were white because of the high density of conidia produced on the colony surface. On 4×UU (20 mmol L^{-1} uridine and 40 mmol L^{-1} uracil) plates, wild type still reproduced mainly by asexual sporulation, while conidial production was dramatically decreased and sexual development remarkably increased in the *pyrG89* mutant, resulting in a yellowish color of colonies (Figures 1 and 2). When observed under a dissecting microscope or by scanning electron microscopy, protocleistothecia, which were surrounded by yellow Hülle cells, were abundant on the surface of the *pyrG89* mutant on 4×UU plates. In contrast, protocleistothecia were rarely seen on the surface of wild type on 4×UU plates and on the surface of the *pyrG* mutant on 1×UU plates (Figure 2).

2.2 *pyrG89* mutation increases sensitivity to excess chemicals related to uracil metabolism

In eukaryotes, cellular uracil can serve as a precursor for synthesis of many other compounds or it can be degraded by two pathways [26,27]. To test whether compounds in-

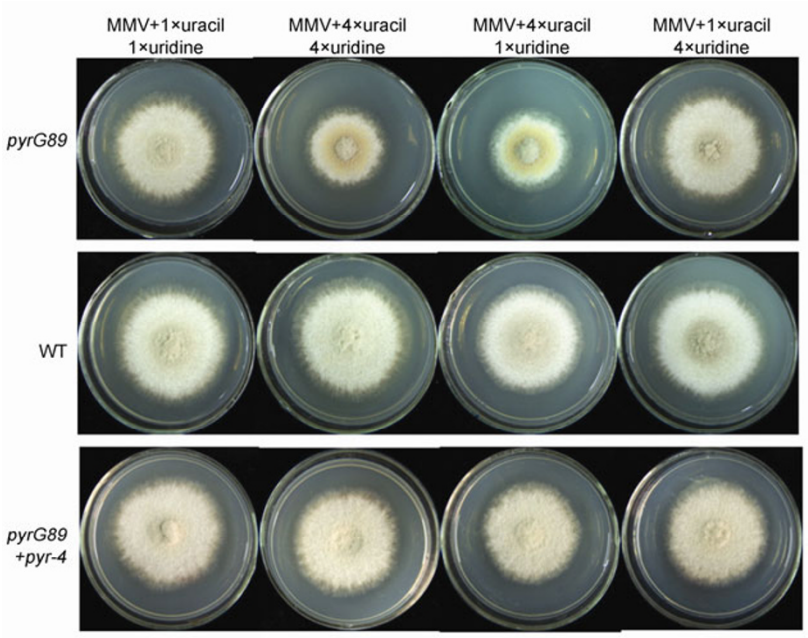


Figure 1 Effects of excess uracil on growth and sexual development of *A. nidulans pyrG89* mutant. Conidia of *A. nidulans* wild type ALS05 (WT), *pyrG89* mutant A773 (p*yrG89*) or complemented strain AHC01 (*pyrG89+pyr-4)* were inoculated onto the center of plates containing MMVUU medium (MMV+1×uridine+1×uracil) (control) or MMVUU medium with 4×additives (4×uridine, or 4×uracil, or 4×uridine and 4×uracil) and incubated at 28°C for 5 d. 5 mmol L^{-1} as 1× for uridine and 10 mmol L^{-1} as 1× for uracil. Experiment was carried out with three replicates.

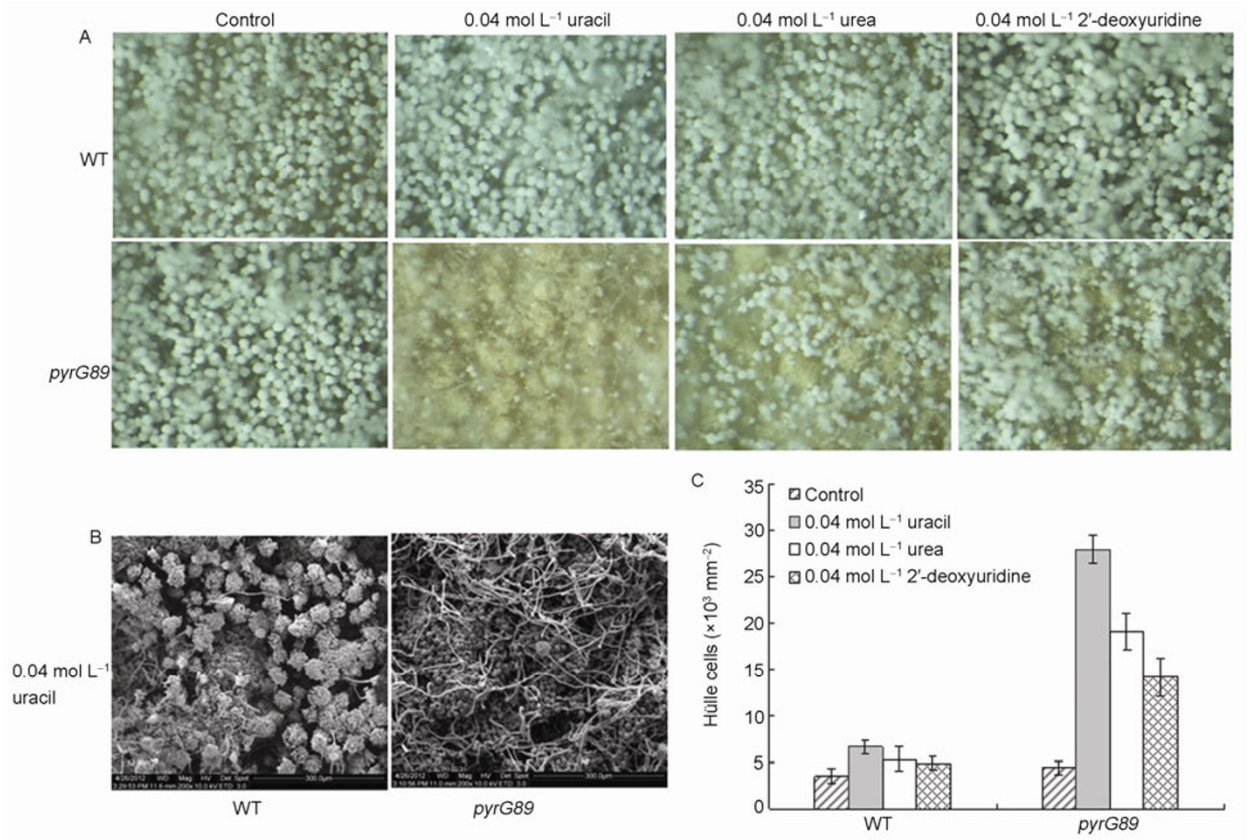


Figure 2 Comparison of sexual differentiation between *A. nidulans* wild type and *pyrG89* mutant in response to excess uracil stress. Conidia of *A. nidulans* wild type or the *pyrG89* mutant were inoculated onto the center of plates containing MMVUU medium (MMV+1×uridine+1×uracil) (control) or MMVUU medium with 4× (0.04 mol L^{-1}) additives (4×uracil, or 4×2′-deoxyuridine, or 4×urea) and incubated at 28°C for 5 d. Images were obtained under a dissecting microscope (A) or a scanning electron microscope (B). Hülle cell production in wild type and the *pyrG89* mutant was compared (C) Number of Hülle cells harvested from colonies was shown as mean values±standard deviation (n=3 replicate dishes). 5 mmol L^{-1} as 1× for uridine and 10 mmol L^{-1} as 1× for uracil, 2′-deoxyuridine or urea.

volved in uracil metabolism have effects similar to uracil, equivalent experiments were conducted using 2′-deoxyuridine (0.04 mol L^{-1}) and urea (0.04 mol L^{-1}) as the stressing chemicals. As shown in Figures 2 and 3, addition of 2′-deoxyuridine (0.04 mol L^{-1}) and urea (0.04 mol L^{-1}) reduced the colony growth of the *pyrG89* mutant but not that of wild type. In addition, 2′-deoxyuridine (0.04 mol L^{-1}) and urea (0.04 mol L^{-1}) induced sexual development of the *pyrG89* mutant but not that of wild type.

2.3 *pyrG89* mutation causes an accumulation of uracil in cells

The hypersensitivity of the *pyrG89* mutant to excess uracil (0.04 mol L^{-1}), 2′-deoxyuridine (0.04 mol L^{-1}), and urea (0.04 mol L^{-1}) is likely a result of its disrupted uracil metabolism. Without a functional PyrG, excess uracil, 2′-deoxyuridine, and urea might accumulate in cells and have toxic effects. To test this hypothesis, cellular uracil, uridine, and AMP in *pyrG89* and wild type grown in minimal medium with 4×uracil and uridine were quantitatively analyzed by HPLC. The cellular uracil, uridine, and AMP levels in the *pyrG89* mutant were 653%, 31%, and 51% higher, respectively, than those in wild type (Figure 4).

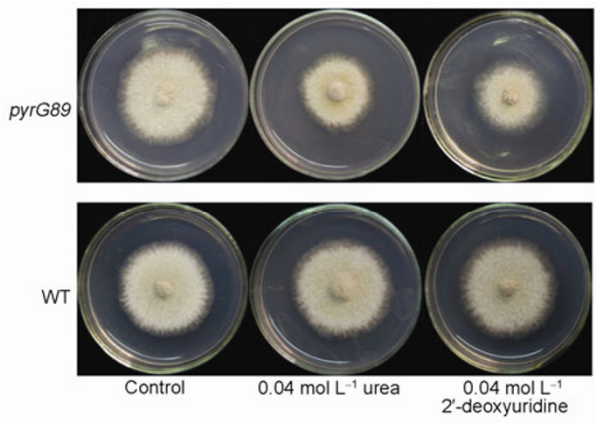
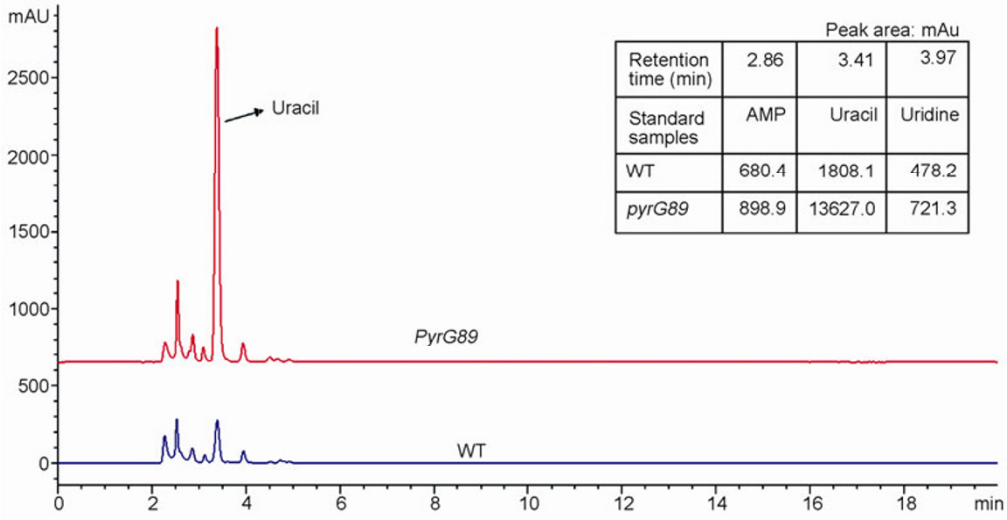


Figure 3 Effects of excess metabolites of uracil on growth and sexual development of *A. nidulans pyrG* mutant. Conidiospores of *A. nidulans* wild type or *pyrG89* mutant were inoculated onto center of plates containing MMVUU medium (MMV+1×uridine+1×uracil) (control) or MMVUU medium with 4× (0.04 mol L^{-1}) additives (4×urea or 4×2′-deoxyuridine) and incubated at 28°C for 5 d. 5 mmol L^{-1} as 1× for uridine and 10 mmol L^{-1} as 1× for uracil, 2′-deoxyuridine, or urea.

2.4 Analysis of transcriptomic responses to excess uracil

To understand the mechanism of uracil accumulation and the developmental effects of uracil on the *pyrG* mutant, we analyzed transcriptomic profiles of the *pyrG89* mutant and wild type grown in liquid medium supplemented with normal (1×) or excess (4×) levels of uracil by DGE profiling. In response to excess uracil stress, 185 and 71 genes were up-regulated in the *pyrG89* mutant and wild type, respectively, and 317 and 162 genes were down-regulated in the *pyrG89* mutant and wild type, respectively. If transcriptional levels of genes in the *pyrG89* mutant and wild type were compared under excess uracil conditions, 1025 genes showed at least 50% lower transcript levels and 655 genes showed higher (>1 fold) transcript levels in the mutant than in wild type (Supplemental Data S1). In response to excess uracil stress, many genes (e.g., ANID_07074, ANID_03925, ANID_10399, ANID_02859, and ANID_05567) showed no transcriptional response in wild type but were dramatically up-regulated in the *pyrG89* mutant (Table 2). Many other genes (e.g., ANID_06657, ANID_01608, ANID_02578, and ANID_05145) maintained high transcript levels in wild



Peak area: mAu

Retention time (min)	2.86	3.41	3.97
Standard samples	AMP	Uracil	Uridine
WT	680.4	1808.1	478.2
pyrG89	898.9	13627.0	721.3

Figure 4 HPLC chromatograms of intermediary metabolites of uracil metabolism in *A. nidulans*. Cellular uracil, uridine, and AMP in *pyrG89* mutant and wild type grown in minimal medium with 4×uracil and 1×uridine were quantitatively analyzed by HPLC. 5 mmol L^{-1} as 1× for uridine and 10 mmol L^{-1} as 1× for uracil.

Table 2 Significant differently transcribed genes between wild type and the *pyrG* mutant in response to excess uracil

Gene[a)]	Function annotation[b)]	TPM[c)]-WT 1×uracil	TPM-*pyrG89* 1×uracil	TPM[c)]-WT 4×uracil	TPM-*pyrG89* 4×uracil	Fold (*pyrG89*/WT) 4×uracil	*P*-value	FDR
Up-regulated genes								
ANID_07074	hypothetical protein, metabolic process and oxidation-reduction process	4.43	14.5	4.87	112.75	23.15	1.70×10^{-11}	1.04×10^{-10}
ANID_03925	hypothetical protein, carbohydrate metabolic process	9.71	44.69	13.11	302	23.04	0	0
ANID_10399	hypothetical protein, oxidation-reduction process	10.39	74.71	9.58	214.52	22.39	5.28×10^{-14}	4.33×10^{-13}
ANID_02859	dihydrodipicolinate synthetase family protein, metabolic process	3.06	17.91	3.19	63.61	19.94	8.44×10^{-9}	4.26×10^{-8}
ANID_08953	alpha-glucosidase B with a predicted role in maltose metabolism, *agdB*	72.37	1748.5	154.45	2911.22	18.85	1.25×10^{-12}	8.37
ANID_09121	*esdC*, sexual development	57.04	221.91	32.77	518.6	15.83	1.40×10^{-13}	1.09×10^{-12}
ANID_05567	GrpB domain protein	4.43	8.19	5.55	82.43	14.85	1.64×10^{-12}	1.09×10^{-11}
ANID_08972	hypothetical protein, transporter activity	3.92	18.76	3.36	45.48	13.54	4.54×10^{-9}	2.34×10^{-8}
ANID_09373	putative Zn(II)2Cys6 transcription factor	20.26	47.59	11.09	139.06	12.54	0	0
ANID_05977	ketoreductase, with role in metabolic process	23.84	65.67	10.42	112.75	10.82	0	0
ANID_10949	ABC transporter protein	2.72	9.89	3.53	32.24	9.13	2.44×10^{-9}	1.28×10^{-8}
ANID_08602	hypothetical protein with oxidoreductase activity	10.9	41.45	18.32	164.85	9.00	0	0
ANID_06669	putative sugar transporter	12.09	97.91	29.41	246.93	8.40	3.08×10^{-13}	2.25×10^{-12}
ANID_05655	5'-nucleotidase (EC:3.1.3.5)	4.43	18.42	3.03	24.4	8.05	1.56×10^{-8}	7.69×10^{-8}
ANID_00841	hypothetical protein	4.6	24.22	4.71	36.94	7.84	3.14×10^{-11}	1.88×10^{-10}
ANID_04220	ankyrin repeat protein, role in meiosis and cytosol, nucleus localization	20.43	41.45	5.55	41.3	7.44	1.64×10^{-12}	1.09×10^{-11}
ANID_02970	4-nitrophenylphosphatase, with role in aminobenzoate degradation	77.13	175	32.6	239.09	7.33	4.61×10^{-13}	3.30×10^{-12}
ANID_03520	hypothetical protein	10.22	40.94	14.45	102.81	7.11	0	0
ANID_10964	methyltransferase, role in metabolic process	19.07	49.64	7.06	49.66	7.03	1.07×10^{-13}	8.47×10^{-13}
ANID_04085	protein phosphotase 2a 65kd regulatory subunit	58.91	114.11	27.56	173.22	6.29	8.93×10^{-14}	7.18×10^{-13}
Down-regulated genes								
ANID_05447	glutamate decarboxylase, amino acid metabolism	395.2	106.95	585.19	18.65	0.032	0	0
ANID_06657	hypothetical protein	1291.17	461.9	1699.6	78.94	0.046	0	0
ANID_01608	hypothetical protein	1406.61	531.32	1732.88	96.19	0.056	0	0
ANID_01510	oxidoreductin	491.06	161.53	802.49	46.53	0.058	0	0
ANID_02578	hypothetical protein with peroxidase activity	3255.76	854.72	4109.27	239.09	0.058	0	0
ANID_05145	hypothetical protein	258.98	88.7	322.51	18.82	0.058	0	0
ANID_04940	protein with role in cellular response to stress	551.68	112.4	728.38	44.79	0.061	0	0
ANID_02555	serine carboxypeptidase, protein metabolism	255.24	79.31	400.99	25.97	0.065	0	0
ANID_12070	glutamyl-tRNA amidotransferase subunit A	237.19	112.92	271.76	17.77	0.065	0	0
ANID_01879	WD repeat protein, role in glutathione catabolic process and cytoplasm, nucleus localization	212.33	47.59	309.91	20.39	0.066	0	0
ANID_04127	hypothetical protein	686.87	209.46	763.84	57.68	0.076	0	0
ANID_11246	nucleobase, nucleoside, nucleotide and nucleic acid transporter	26.39	5.97	50.59	3.83	0.076	7.01×10^{-62}	1.26×10^{-60}
ANID_04135	delta-9-stearic acid desaturase, fatty acid metabolism	212.5	67.89	367.38	28.23	0.077	0	0
ANID_10299	hypothetical protein, amino acid metabolism	245.02	103.71	439.14	33.98	0.077	0	0
ANID_02286	alcohol dehydrogenase III (ADH III)	515.07	223.1	484.69	39.91	0.082	0	0
ANID_08122	MFS multidrug transporter	34.91	9.72	60	5.05	0.084	1.07×10^{-70}	2.05×10^{-69}
ANID_01378	hypothetical protein	1117.66	840.39	1519.27	132.26	0.087	0	0
ANID_09297	67 kDa myosin-cross-reactive antigen family protein	454.28	183.36	548.89	48.44	0.088	0	0
ANID_06525	NAD-dependent formate dehydrogenase (FDH)	335.43	148.05	473.26	43.22	0.091	0	0
ANID_00186	hypothetical protein	148.14	59.7	283.35	25.97	0.092	0	0
ANID_03796	hypothetical protein	145.07	78.12	208.56	19.34	0.093	1.46×10^{-233}	4.10×10^{-232}
ANID_08461	hypothetical protein	234.46	220.55	281.5	28.06	0.100	4.00×10^{-306}	1.23×10^{-304}
ANID_09470	uricase (urate oxidase), purine metabolism	338.84	136.63	450.4	44.96	0.100	0	0

a) Gene accession numbers were annotated according to *Aspergillus* comparative genome database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html/). b) Function annotations were obtained from broad description of genes. c) TPM, tags per million.

type but not in the mutant under uracil stress. These findings indicate that the *pyrG89* mutant had more significant transcriptional responses to uracil stress than did wild type, suggesting that the *pyrG89* mutant suffered greater stress than did wild type under these conditions. The maintenance of stable gene expression levels might be important for cellular metabolism and growth under uracil stress.

2.5 Transcriptional responses by nucleotide metabolism-related genes to excess uracil

Gene set enrichment analysis showed that differentially transcribed genes between the two tested strains were mainly involved in C-compound and carbohydrate metabolism, transportation of compounds, and functions related to disease, virulence, and defense (Table S1). Among the genes directly related to uracil metabolism, ANID_05655, which encodes a 5′-nucleotidase (EC: 3.1.3.5), showed an 8-fold higher transcript level in the *pyrG89* mutant than in wild type (Table 2). 5′-nucleotidase catalyzes the dephosphorylation of nucleoside monophosphates and is involved in salvaging preformed nucleotides [28]. The increased transcript levels of the 5′-nucleotidase gene suggest that the balance between nucleotides and nucleosides is disordered in the mutant.

Transport is the first step for salvage of exogenous pyrimidine bases. In *A. nidulans*, *furD* (ANID_11247) and *cntA* (ANID_05493) are uracil- and uridine-transporter genes, respectively [29]. As shown in Table 3 and Table S2, neither of these genes showed higher transcriptional levels in the *pyrG* mutant than in wild type.

2.6 Transcriptional responses of sexual development-related genes to excess uracil

In response to excess uracil, several genes involved in sexual development were differentially transcribed in the *pyrG89* mutant relative to wild type. As shown in Table 3, transcript levels of *esdC* (ANID_09121), *nosA*, and *noxA* in the *pyrG89* mutant were more than 4-fold higher than in wild type. Conversely, transcript levels of the flavohemoglobin gene *fhbA* in the *pyrG89* mutant were only 26% of that in wild type. These results were confirmed by qRT-PCR (Figure 5).

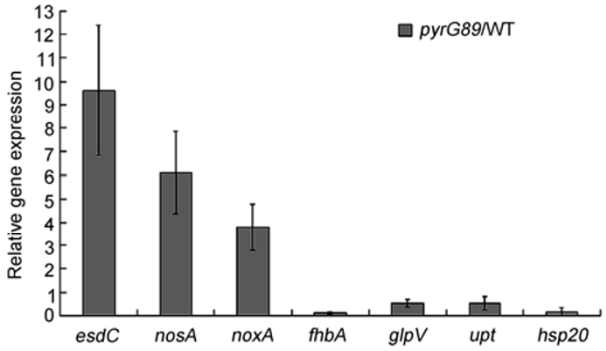


Figure 5 Differential transcriptions of genes in *pyrG89* mutant relative to wild type as determined by qRT-PCR. Relative transcriptional levels of four down-regulated genes and three up-regulated genes are shown. Values shown are means of three independent replicates±standard deviation.

Table 3 Comparison of transcriptional responses of uracil transporter genes and genes involved in sexual development between wild type and pyrG mutant grown in medium with excess uracil

Gene[a)]	Function annotation[b)]	TPM[c)]-WT 1×uracil	TPM-*pyrG89* 1×uracil	TPM[c)]-WT 4×uracil	TPM-*pyrG89* 4×uracil	Fold (*pyrG89/WT*) 4×uracil	*P*-value	FDR
Uracil and uridine transporter genes								
ANID_11247	uracil transporter FurD	0.34	0.01	0.84	0.01	—[d)]	0.0348	0.0688
ANID_05493	Concentrative nucleoside permease CntA	3.41	3.75	5.71	5.05	—	0.6319	0.7132
The genes involved in sexual development								
ANID_01052	VeA	72.54	102.17	82.35	134.17	1.63	0	0
ANID_03152	sexual development transcription factor NsdD	1.7	0.68	0.34	0.01	—	0.2638	0.3483
ANID_04263	C2H2 zinc finger protein NsdC	0.85	1.02	1.01	0.35	—	0.1982	0.2944
ANID_09121	early sexual development EsdC	57.04	221.91	32.77	518.60	15.83	1.40×10^{-13}	1.09×10^{-12}
ANID_06505	transcriptional repressor rco-1, TupA/RocA	366.76	345.23	402.34	455	—	1.37×10^{-5}	5.03×10^{-5}
ANID_05170	sexual development transcription factor RosA	0	0	0	0	—	—	—
ANID_02260	NADH-ubiquinone oxidoreductase subunit	403.2	383.27	578.97	346.78	0.60	1.23×10^{-76}	2.40×10^{-75}
ANID_01848	C6 sexual development transcription factor NosA	3.41	3.24	1.18	5.92	5.02	1.95×10^{-5}	7.03×10^{-5}
ANID_02330	late sexual development protein	0	0	0	0	—	—	—
ANID_07169	flavohemoglobin gene *fhbA*	2222.72	1699.55	1077.95	276.55	0.26	0	0
ANID_00807	methyltransferase LaeA	0.01	0.68	1.18	0.01	0.01	0.0090	0.0213
ANID_05457	NADPH oxidases NoxA	4.43	13.82	5.04	29.62	5.88	9.27×10^{-12}	5.79×10^{-11}
ANID_05836	cell pattern formation-associated protein StuA	5.96	5.8	4.54	5.92	—	0.3022	0.3899

a) Gene accession numbers were annotated according to *Aspergillus* comparative genome database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html/). b) Function annotations were obtained from broad description of genes. c) TPM, tags per million. d) "—" indicates *P*-value greater than 0.01.

The *A. nidulans esdC* (early sexual development) gene is necessary for sexual development [30]. The *nosA* gene controls fruiting body formation in *A. nidulans* [31]. The *noxA* gene is induced during sexual development, and deletion of *noxA* specifically blocks differentiation of sexual fruit bodies (cleistothecia) [32]. For the flavohemoglobin gene *fhbA*, deletion of *fhbA* induced sexual development [33]. Thus, the up-regulation of *esdC*, *nosA* and *noxA* genes and down-regulation of *fhbA* in the *pyrG* mutant theoretically favors sexual development.

3 Discussion

3.1 *PyrG* participates in maintaining steady levels of cellular uracil

OMP decarboxylase is required for pyrimidine synthesis. Cellular uracil levels are tightly controlled to prevent the accumulation of uracil. This is accomplished by the regulation of uracil permeases at transcriptional, posttranscriptional, and posttranslational levels [13–15]. In this study, we found that excess uracil in the medium caused uracil accumulation in cells of the *pyrG89* mutant but not those of wild type, indicating that OMP decarboxylase participates in maintaining cellular uracil at a low level. In *A. nidulans*, *furD* (ANID_11247) and *cntA* (ANID_05493) are the only known transporter genes for uracil and uridine, respectively. It was proposed that more uracil transporter genes should exist in this fungus [29]. Thus, although *furD* and *cntA* did not display statistically higher transcript levels in the *pyrG* mutant than in wild type, we cannot exclude the possibility that accumulation of cellular uracil in the *pyrG89* mutant is due to increased uracil transport. If the accumulation of uracil is independent of uracil transport, one possible explanation is that PyrG might play a role in recycling extra uracil by an unknown mechanism, in addition to its role in catalyzing UMP biosynthesis.

Excess accumulation of uracil is believed to be toxic to cells, but these toxic effects were not observed. Here, we provide direct evidence that high concentrations of uracil affect the growth and development of *A. nidulans*. Because they can accumulate high levels of uracil, *pyrG89* mutants would be useful research materials to investigate whether increased cellular uracil can elevate the frequency of uracil incorporation into DNA. In contrast to uracil, uridine did not have inhibitory effects, even though it accumulated in the *pyrG89* mutant. Since uridine cannot be directly incorporated into DNA as a substrate to synthesize uracil-DNA, it might be less toxic than uracil.

3.2 Excess uracil activates sexual development

For most filamentous ascomycetes, asexual sporulation is the major reproduction mode under normal conditions, while sexual development is usually induced by extreme conditions such as low temperature or extended darkness. Therefore, activation of sexual development might be a strategy for fungi to survive under extremely unfavorable conditions. Here, we showed that uracil and other metabolites involved in uracil metabolism could activate sexual development and suppress conidiation in the *pyrG89* mutant, and that the transcriptions of genes involved in sexual development, such as *esdC*, *nosA*, and *noxA*, could be induced by uracil. These observations suggest that cellular accumulation of uracil and related metabolites can alter the developmental pattern of *A. nidulans*. Similar to the *pyrG89* mutant grown under excess uracil stress, *A. nidulans* mutants with defects in sphingolipid biosynthesis displayed enhanced sexual sporulation and reduced conidial production [34]. These phenomena indicate that sexual development can be activated by diverse metabolic abnormalities. Each metabolic process might have monitors to sense changes. However, abnormal signals from different metabolic monitors might be passed to the same unknown signaling pathway that determines developmental fate. Activated by metabolic monitors, this pathway might transcriptionally activate *esdC*, *nosA*, and *noxA*, which further activate expressions of genes required for sexual development.

3.3 *pyrG* affects sensitivity to multiple stresses

Auxtotrophic *pyrG* mutants are the most frequently used parent strains for generating new strains by gene deletion or overexpression in *A. nidulans* and other fungal species. Phenotypic observations of new strains derived from *pyrG* mutants are often conducted using the parental *pyrG* mutants as controls. However, this study clearly demonstrates that the *pyrG* mutation can alter the growth and developmental pattern. Thus, when new mutants are generated using a *pyrG* mutant as the parent, care must be taken to ensure that the resulting phenotypes are due to the new mutation alone, and do not reflect the *pyrG* mutation.

This work was supported by the National Natural Science Foundation of China (31000551 to Sun XianYun, 31170087 to Li ShaoJie) and a grant from the "100 Talent Program" from Chinese Academy of Sciences to Li ShaoJie. We thank Prof. Liu Gang and Dr. Wu Sheng (Institute of Microbiology, Chinese Academy of Sciences) for valuable discussion about the experimental design and Prof. Nick D. Read (University of Edinburgh, UK) for proof reading of the manuscript.

1 Bach M L, Lacroute F. Direct selective techniques for the isolation of pyrimidine auxotrophs in yeast. Mol Gen Genet, 1972, 115: 126–130
2 Palmer L M, Cove D J. Pyrimidine biosynthesis in *Aspergillus nidulans*: isolation and preliminary characterisation of auxotrophic mutants. Mol Gen Genet, 1975, 138: 243–255
3 Perkins D D, Radford A, Newmeyer D, et al. Chromosomal loci of *Neurospora crassa*. Microbiol Rev, 1982, 46: 426–570
4 Boeke J, LaCroute F, Fink G R. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet, 1984, 197: 345–346
5 Ballance D J, Turner G. Development of a high-frequency trans-

forming vector for *Aspergillus nidulans*. Gene, 1985, 36: 321–331
6 Díez B, Alvarez E, Cantoral J M, et al. Selection and characterization of *pyrG* mutants of *Penicillium chrysogenum* lacking orotidine-5′-phosphate decarboxylase and complementation by the *pyr4* gene of *Neurospora crassa*. Curr Genet, 1987, 12: 277–282
7 van Hartingsveldt W, Mattern I E, van Zeijl C M, et al. Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol Gen Genet, 1987, 206: 71–75
8 Smith J L, Bayliss F T, Ward M. Sequence of the cloned *pyr4* gene of *Trichoderma reesei* and its use as a homologous selectable marker for transformation. Curr Genet, 1991, 19: 27–33
9 Weidner G, d'Enfert C, Koch A, et al. Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5′-monophosphate decarboxylase. Curr Genet, 1998, 33: 378–385
10 Oakley B R, Rinehart J E, Mitchell B L, et al. Cloning, mapping, and molecular analysis of the *pyrG* (orotidine-5′-phosphate decarboxylase) gene of *Aspergillus nidulans*. Gene, 1987, 61: 385–399
11 Waring R B, May G S, Morris N R. Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. Gene, 1989, 79: 119–130
12 Chevallier M R. Cloning and transcriptional control of a eucaryotic permease gene. Mol Cell Biol, 1982, 2: 977–984
13 Galan J M, Moreau V, André B, et al. Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. J Biol Chem, 1996, 271: 10946–10952
14 Séron K, Blondel M O, Haguenauer-Tsapis R, et al. Uracil-induced down regulation of the yeast uracil permease. J Bacteriol, 1999, 181: 1793–1800
15 Amillis S, Hamari Z, Roumelioti K, et al. Regulation of expression and kinetic modeling of substrate interactions of a uracil transporter in *Aspergillus nidulans*. Mol Membr Biol, 2007, 24: 206–214
16 Verri A, Mazzarello P, Biamonti G, et al. The specific binding of nuclear proteins to the cAMP responsive element sequence is reduced by misincorporation of uracil and increased be the deamination of cytosine. Nucleic Acids Res, 1990, 18: 5775–5780
17 Miller B, Miller K, Timberlake W. Direct and indirect gene replacements in *Aspergillus nidulans*. Mol Cell Biol, 1985, 5: 1714–1721
18 Calvo A M, Bok J, Brooks W, et al. *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. Appl Environ Microbiol, 2004, 70: 4733–4739
19 Käfer E. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet, 1977, 19: 33–131
20 Ruijter G J G, Visser J. Determination of intermediary metabolites in *Aspergillus niger*. J Microbiol Methods, 1996, 25: 295–302
21 Nielsen K, Høgh A L, Emmersen J. DeepSAGE-digital transcriptomics with high sensitivity, simple experimental protocol and multiplexing of samples. Nucleic Acids Res, 2006, 34: e133
22 Sun X Y, Zhang H X, Zhang Z Y, et al. Involvement of a helix-loop-helix transcription factor CHC-1 in CO_2-mediated conidiation suppression in *Neurospora crassa*. Fungal Genet Biol, 2011, 48: 1077–1086
23 Audic S, Claverie J M. The significance of digital gene expression profiles. Genome Res, 1997, 7: 986–995
24 Benjamini Y, Drai D, Elmer G, et al. Controlling the false discovery rate in behavior genetics research. Behav Brain Res, 2001, 125: 279–284
25 Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. Methods, 2001, 25: 402–408
26 Vogels G D, Van der Drift C. Degradation of purines and pyrimidines by microorganisms. Bacteriol Rev, 1976, 40: 403–468
27 Andersen G, Björnberg O, Polakova S, et al. A second pathway to degrade pyrimidine nucleic acid precursors in eukaryotes. J Mol Biol, 2008, 380: 656–666
28 Hunsucker S A, Mitchell B S, Spychala J. The 5′-nucleotidases as regulators of nucleotide and drug metabolism. Pharmacol Ther, 2005, 107: 1–30
29 Hamari Z, Amillis S, Drevet C, et al. Convergent evolution and orphan genes in the Fur4p-like family and characterization of a general nucleoside transporter in *Aspergillus nidulans*. Mol Microbiol, 2009, 73: 43–57
30 Han K H, Kim J H, Moon H, et al. The *Aspergillus nidulans esdC* (*early sexual development*) gene is necessary for sexual development and is controlled by *veA* and a heterotrimeric G protein. Fungal Genet Biol, 2008, 45: 310–318
31 Vienken K, Fischer R. The Zn(II)2Cys6 putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans*. Mol Microbiol, 2006, 61: 544–554
32 Lara-Ortíz T, Riveros-Rosas H, Aguirre J. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Mol Microbiol, 2003, 50: 1241–1255
33 Baidya S, Cary J W, Grayburn W S, et al. Role of nitric oxide and flavohemoglobin homolog genes in *Aspergillus nidulans* sexual development and mycotoxin production. Appl Environ Microbiol, 2011, 77: 5524–5528
34 Li S, Bao D, Yuen G, et al. *basA* regulates cell wall organization and asexual/sexual sporulation ratio in *Aspergillus nidulans*. Genet, 2007, 176: 243–253

Supporting Information

Table S1 Differentially transcribed genes grouped by functional classification and gene set enrichment analysis (4×uracil, Δ*pyrG89*/WT)

Table S2 Differentially transcribed genes between wild type and the *pyrG* mutant in response to excess uracil

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