

# Isolation and Differential Expression of *ura5* Gene during Phase Transition and Stress Conditions in a Human Pathogenic Dimorphic Fungus, *Penicillium marneffei*

## การแยก และแสดงออกของยีน *ura5* ในระหว่างที่มีการเปลี่ยนรูป และสถานะที่มีความเครียด ในเชื้อราสองรูปที่ก่อโรคในมนุษย์ เพนนิซิเลียม มาร์เนฟฟีไอ

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### ABSTRACT

*Penicillium marneffei*, a dimorphic fungus, can cause an opportunistic infection in immunocompromised patients especially in AIDS patients. Molecular genetic studies of *P. marneffei* provide more understanding about the mechanism of fungal pathogenesis. We have isolated and characterized the *ura5* gene, which encodes orotate phosphoribosyltransferase (OPRTase), from *P. marneffei*. The nucleotide and amino acid sequences of *ura5* displayed strong homology to OPRTase proteins in other fungi. Analysis of the *ura5* gene expression by RT-PCR revealed that the expression was upregulated at an early step during mycelium to yeast phase transition, and substantially upregulated in mycelial cells exposed to 39°C or treated with 1 mM of H<sub>2</sub>O<sub>2</sub> for 30 min. The results suggested that the *ura5* gene might play some role on the thermal adaptation of *P. marneffei* during temperature-induced yeast phase transition and stress conditions.

### บทคัดย่อ

เชื้อเพนนิซิเลียมมาร์เนฟฟีไอจัดเป็นราสองรูปที่สามารถก่อให้เกิดการติดเชื้อแบบฉวยโอกาสในผู้ป่วยที่มีภูมิคุ้มกันบกพร่องโดยเฉพาะผู้ป่วยเอดส์ การศึกษาในระดับโมเลกุลของเชื้อนี้จะทำให้มีความเข้าใจต่อกลไกการก่อโรคได้มากขึ้น ในการศึกษาครั้งนี้ได้ทำการแยก และศึกษาคุณลักษณะของยีน *ura5* โดยยีนนี้กำหนดการสร้างโปรตีน orotate phosphoribosyltransferase (OPRTase) ผลการศึกษาพบว่า ลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนที่ได้มีความเหมือนกับโปรตีน OPRTase ของเชื้อราอื่นอย่างมาก ในการวิเคราะห์การแสดงออกของยีน *ura5* โดยใช้วิธี RT-PCR พบว่า การแสดงออกของยีนนี้จะเพิ่มขึ้นในช่วงแรกในระหว่างที่มีการเปลี่ยนรูปร่างของเชื้อจากสาหร่ายไปเป็นรูปยีสต์ และนอกจากนี้ยังพบการแสดงออกของยีนที่เพิ่มขึ้นอย่างมากในสถานะที่นำ

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เชื้อในรูปของสายรามาลีงที่อุณหภูมิ 39°C หรือ นำมาเลี้ยงในอาหารที่มี  $H_2O_2$  เข้มข้น 1 mM เป็นเวลา 30 นาที ผลการศึกษาในครั้งนี้แสดงให้เห็นว่า ยีน *ura5* อาจจะมีบทบาทต่อการปรับตัวของเชื้อต่ออุณหภูมิในระหว่างที่เชื้ออยู่ในอุณหภูมิที่เหนียวทำให้เชื้อมีการเปลี่ยนไปเป็นยีสต์ และในขณะที่เชื้ออยู่ในสภาวะเครียด

**Key Words :** *Penicillium marneffeii*, Dimorphic fungus, Stress condition

**คำสำคัญ :** เพนนิซิลเลียมมาร์เนฟฟีโอ ราสองรูป สภาวะเครียด

## Introduction

*Penicillium marneffeii* (*P. marneffeii*) is the only dimorphic species of the genus *Penicillium*. It is the etiological agent of penicilliosis marneffeii, a disease involving infection of the reticuloendothelial system, and rarely noted before the epidemic of the acquired immune deficiency syndrome (AIDS). The fungus always disseminates in immunocompromised patients and, if untreated, leads to death. The infection has become more prevalent in the endemic areas that encompass in Southeast Asia and Southern China, paralleling with the onset of HIV infection (Supparatpinyo *et al.*, 1994; Vanittanakom *et al.*, 2006; Cooper and Vanittanakom, 2008). Genetic studies of *P. marneffeii* have been frequently documented since 2000. Although a number of genetic studies involving *P. marneffeii* morphogenesis have been reported, only a few genes have been characterized to play some role in the regulation of dimorphism (Cooper and Vanittanakom, 2008). More studies will be needed for understanding the pathogenesis of this fungus.

It has been demonstrated for several bacterial and fungal pathogens that organisms with mutations involving components of the purine or pyrimidine biosynthetic pathway are less virulent in cultured cells or whole animals (Buchmeier and Libby, 1997; Shepherd, 1985; Retallack *et al.*, 1999). In *P.marneffeii*, the isolation and characterization of the *ura5* gene has not been reported. Therefore, the purpose of this study was

to isolate and characterize the *ura5* gene from this fungus. In addition, gene expression pattern was observed during phase transition and stress conditions.

## Materials and Methods

### 1. Strains and growth conditions

*P. marneffeii* F4 (CBS no. 119456) was obtained from the hemoculture of an AIDS patient from the Central Laboratory, Maharaj Nakorn Chiang Mai Hospital in January 1999. The primary isolate was kept in 20% glycerol at -80°C. The fungus was grown on malt extract agar (OXOID, England) for 7 days at 25°C. A conidial suspension for inoculating broth cultures was prepared by cotton swab-scraping in sterile normal saline and filtrating through sterile glass wool (Corning, Acton, MA, USA). The conidia were counted using a hemocytometer. Approximately  $10^7$  conidia were cultured in 50 ml brain-heart infusion (BHI) broth cultures at 25°C and 37°C for 96 h to produce mycelial and yeast forms, respectively. To investigate mycelium to yeast phase transition, one milliliter of complete mycelial cells in BHI broth (25°C, 72 h) was transferred to fresh BHI broth and incubated at 37°C for 6, 12, 24, 48, and 72 h. To generate the heat shock condition, yeast cells (37°C, 72 h) or mycelial cells in BHI were subjected to 39°C heat for 30 min. To generate the  $H_2O_2$ -treated condition, hydrogen peroxide was added into flasks containing yeast or mycelial

cells to a final concentration of 1 or 2 mM and incubated for 30 min at either 37°C or 25°C. All cultures were maintained in shaking incubator with continuous shaking at 150 rpm. For the macrophage infection experiment, conidia were co-cultured with macrophages or inoculated in cell-free medium (Thirach *et al.*, 2007). The infected macrophages were harvested at the desired time by centrifugation at 12,000 rpm for 5 min.

## 2. Genomic DNA and RNA extraction

Approximately  $10^8$  harvested conidia were inoculated into Sabouraud dextrose broth (SDB) and incubated at 25°C in water bath

shaking at 150 rpm for 12 h to get germinated form of conidia. Fungal genomic DNA was isolated by using a modified method (Vanittanakom *et al.*, 1996). DNA quantity was determined by using spectrophotometer (Eppendorf, Hamburg, Germany) and by gel electrophoresis.

Total RNA was isolated from *P. marneffei* cells by mechanical disruption using 0.5 mm glass beads in a mini bead beater (Biospec, Oklahoma, USA). Total RNA isolation was carried out following the manufacturer's protocol of RNeasy mini kit (Qiagen GmbH, Germany).

| Pmura5-5 |                                                                      |
|----------|----------------------------------------------------------------------|
| 1        | <u>ctcgtgactcttgcatctcagctttgatccttaccgctacaagcgattcgctcttataaa</u>  |
|          | M A T P Q S A D Q E D Y K T N                                        |
| 61       | <u>ccccgagatcctcaaacATGGCTACCCCTCAATCCGCTGATCAGGAGGACTACAAGACCAA</u> |
|          | L L S L L I A N N A L A F G T F T L K S                              |
| 121      | <u>TCTCCTTTTCGCTGCTCATAGCCAACAATGCGCTTGCATTGGAACTTTCACATTGAAGTC</u>  |
|          | G R E S P Y F L T S S R L Y T A P L L R                              |
| 181      | <u>CGGTCGCGAGTCGCGTACTTTTGGACCTCGAGTCGTCCTCTATACCGCCCTCTGTTGCG</u>   |
|          | Q V S A A F A N T I S S P P F V N I A A                              |
| 241      | <u>CCAGGTTTCAGCCGCTTCGCAAATACTATCTCTCCCGCCTTTTGTGAATATAGCTGC</u>     |
|          | D G A I T P N F D I V F G                                            |
| 301      | <u>CGACGGCGCTATCACCCGAACTTCGACATTGTTTTTGGgtatgtaccatatcatttgga</u>   |
|          | P A Y K G I P E                                                      |
| 361      | <u>taatgtatgcgccagttgcctgaagtaattgataTCCCGCTACAAAGGAATCCCAGAA</u>    |
|          | C V G V V N E L A T R D A L A G T K T W                              |
| 421      | <u>TGCGTTGGTTCGTCACGAACCTCGCTACCCGGGACGCGCTTGCCGGCACAAGACATGG</u>    |
|          | D N I S Y S F N R K E A K D H G E G G N                              |
| 481      | <u>GATAACATCAGCTACTCCTTCAACCGTAAAGAAGCCAAAGACCACGGTGAGGGCGGTAAT</u>  |
|          | I V G A P L K G K R V L I V D D V I T A                              |
| 541      | <u>ATCGTCGGAGCGCCCTCAAGGGCAAGCGCTTTTGATCGTTGACGACGTCATCACCGCT</u>    |
|          | G T A L R E A V G I I Q K E G G V V A G                              |
| 601      | <u>GGTACCGCGCTCCGTGAAGCTGTTGGAATCATTGAGAAGGAAGGTGGAGTCGTTGCTGGT</u>  |
|          | V V L L F D R Q E R V S D T E Q K S A I                              |
| 661      | <u>GTTGTTTTGCTGTTTGATCGCCAGGAAGAGTCAGTGATACCGAACAGAAGAGCGCTATT</u>   |
|          | G A A E R D L G G D I P I R A V L V L Q                              |
| 721      | <u>GGAGCCGCGGAGAGGATCTTGGAGGAGATATCCCATCCGTGCGGTGTTGGTGCTCCAG</u>    |
|          | D L I D K L G D K I G Q E E V R R L E E                              |
| 781      | <u>GATTTGATTGATAAGCTTGGAGATAAGATCGGCCAGGAGGAAGTGCAGGTTGGAAGAG</u>    |
|          | Y R N K Y K A Q *                                                    |
| 841      | <u>TACCGAAACAAATACAAGGCCAGTAGaaagctgccattgtggacaagatg</u>            |
|          | Pmura5-3                                                             |

**Figure 1** The nucleotide sequence and deduced amino acid sequence of the *ura5* gene from *Penicillium marneffei*. The exon is indicated by uppercase letters, while the introns, 5' and 3' non-translated nucleotides are indicated by lowercase letters. Base numbers are on the left, and amino acid numbers are on the right. Nucleotides in underline and italics represent the conserved 5' and 3' consensus of the introns. Binding sites of primers for isolating *ura5* gene from genomic DNA are underlined.

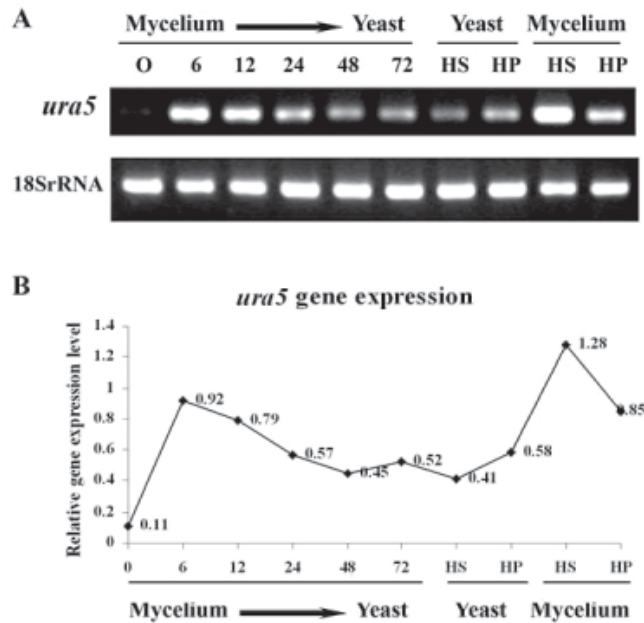


**Figure 2** Differential gene expression during phase transition and stress-induced conditions. RT-PCR was performed by using total RNA extracted from fungal cells during the temperature-induced morphological transition of the fungus, heat shock, and  $H_2O_2$ -treated condition. Mycelium (25°C, 72 h) and cells encountered to yeast-inducible temperature (37°C) for 0 to 72 h.; HS: yeast cells (37°C, 72 h) exposed to heat shock (39°C) for 30 min.; yeast cell treated with 1 mM  $H_2O_2$  (HP) for 30 min. Panel A shows the results of gel electrophoresis analysis. Panel B shows the semi-quantitative analysis of *ura5* expression, performed by densitometry. Relative expression levels were calculated in relation to the internal control 18S rRNA.

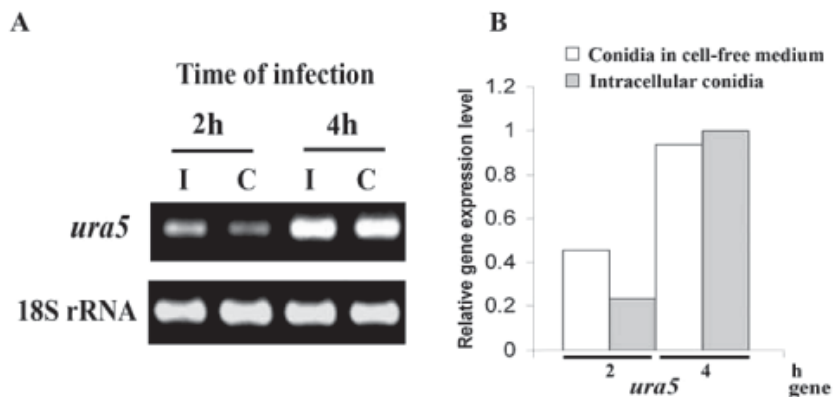


**Figure 3** Expression of *P. marneffei ura5* gene during macrophage infection. After co-culturing *P. marneffei* conidia with murine macrophage cells J774.1 for 2 and 4 h, the fungal cells were harvested by centrifugation after macrophage lysis, and designated as “I”. Total RNA extraction and RT PCR were performed as described in the materials and methods section. Total RNA from *P. marneffei* conidia cultivated *in vitro*, in cell-free medium for 2 and 4 h, was used as control and designated as “C”. Panel A shows the results of gel electrophoresis analysis. Panel B shows the semi-quantitative analysis of *ura5* expression, performed by densitometry. Relative expression levels were calculated in relation to the internal control 18S rRNA.

### 3. Isolation of *ura5* cDNA and *ura5* genomic DNA of *P. marneffei* F4

To obtain *ura5* fragments from *P. marneffei* genomic DNA, PCR amplification using degenerate primers was performed. The primers were designed from multiple sequence alignments on the basis of identity/similarity to amino acid sequence of the other fungi, considering the high similarity conserved region. To amplify a DNA fragment of each gene, approximately 100 ng of *P. marneffei* genomic DNA was subjected to the following reaction: 100 µM of each degenerate primers; 250 µM dNTP; 1x PCR buffer; 3 mM MgCl<sub>2</sub> and 2U *Taq* DNA polymerase in the final volume of 25 µl. PCR amplification conditions were 5 min for denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, annealing temperature for a pair of primers Pmura5F (5'-TTYGGICIGCITAYAARGG-3') and Pmura5R (5'-CCICCYTCICCRTGIBCYTT-3') at 61°C for 1 min, 72°C for 2 min, and a final extension step of 72°C for 10 min. The PCR products were cloned into pTZ57R/T (Fermentas) cloning vector and transformed into *E. coli* DH5α competent cells. Plasmids containing PCR products were subjected to sequencing. Analyzed-fragments of *ura5* gene were used as probes in cDNA library screening to obtain full length cDNAs. The cDNA library of *P. marneffei* yeast phase was constructed using a system of SuperScript Lambda ZipLox vector (Gibco BRL) (Pongpom *et al.*, 2005). The procedure of DNA hybridization and detection were carried out by following the directions of the ECL Direct Nucleic Acid Labeling and Detection Kit (Amersham). The complete genomic sequence of *ura5* gene was obtained by PCR amplification of the genomic DNA of *P. marneffei*. Primers were designed based on the cDNA sequence. A 891 bp PCR product covering the open reading frame of *ura5* was obtained by using the sense primer,

Pmura5-5 (5'-CTCGTGACTCTTGCATCTCA-3'), antisense primer, Pmura5-3 (5'-CATCTTGTCCACAATGGCA-3') (Fig.1). The PCR condition was performed with 100 ng of genomic DNA of *P. marneffei* F4 by using Phusion™ Hot Start High-Fidelity DNA Polymerase (New England Biolabs-Finnzyme). The amplification conditions were started at 98°C for 30 second; followed by 35 cycles at 98°C for 30 s, 60°C for 30 s, 72°C for 30 second; and a final extension at 72°C for 7 min. An amplified PCR product was directly subjected to cloning into pJET1.2/blunt vector (Fermentas) and sequenced.

### 4. Sequencing, sequence analysis

DNA sequencing of the cDNA was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The NCBI BLAST program (<http://www.ncbi.nlm.nih.gov>) was used to search for nucleotide and protein sequence similarities. The program, "proteomics and sequence analysis tools" (<http://www.expasy.org/>), was used to predict an open reading frame and deduced amino acid sequences from nucleotide sequences. The nucleotide sequence of the *ura5* gene from cDNA clone was submitted to the GenBank database under the accession number HQ231917. Deduced amino acid sequences of *P. marneffei ura5* gene and other fungal Ura5 sequences obtained from the GenBank, were used in multiple alignment. Multiple sequence alignment was generated by the ClustalW program (<http://www.ebi.ac.uk/clustal-w/>).

### 5. Analysis of genes expression during phase transition and stress conditions

The expression pattern of the *ura5* gene was investigated using the RT-PCR method. The RNA samples from *P. marneffei* in different phases as well as in intracellular and control conidia (2 and 4 h of incubation), were amplified with

duplex-specific primers using the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. One hundred ng of DNaseI-treated total RNA was used as the template for RT-PCR. Primers for RT-PCR were designed as specific for *ura5* gene; URA5Fexp (5'-GATCAGGAGGACTAC AAGACCA-3') and URA5Rexp (5'-GCGCTC TTCTGTTCTGGTATCA-3'). The internal control consisted of a 630 bp PCR product that was amplified with specific primers for 18S rRNA; RRF1 and RRH1 (Vanittanakom *et al.*, 1998). Reverse transcription was performed at 50°C for 30 min, followed by an initial PCR step at 95°C for 15 min. The subsequent 25 cycles of PCR amplification were performed at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. A PCR without RT was performed to detect DNA contamination in RNA samples. Band intensity of the RT-PCR products was analyzed using a program in GelDoc1000 (BIO-RAD, Hercules, CA, USA). To observe the differential expression, a relative expression level of expressed genes was calculated from the ratio of band intensity between the *ura5* and 18S rRNA control genes.

## Results

### 1. Isolation and sequencing of the *ura5* gene

A 150-bp fragment was amplified by means of PCR with genomic DNA of *P. marneffei* F4 as template using a pair of degenerate primers. The amino acid sequence of the predicted translation product showed high similarity to orotate phosphoribosyltransferase (OPRTase) proteins from other fungi. The obtained full-length *ura5* cDNA clone was 835 nucleotides in length. An open reading frame of *ura5* was composed of 735 nucleotides. The polypeptide of 244 amino acids had a calculated molecular mass of 26.31 kDa and

a pI of 5.40. In order to find out the presence of intron sequence in the *P. marneffei ura5* gene, a genomic fragment corresponding to the entire region of the gene was isolated by PCR, using Pmura5-5 and Pmura5-3 primers (Figure 1). Comparison of the *ura5* cDNA and genomic DNA sequences revealed one intron of 56 nucleotides. Their 5' and 3' ends conformed to the basic consensus GT/AG for eukaryotic splice donor and acceptor sites (Breathnach and Chambon, 1981; Mount, 1982). Alignment of the predicted *P. marneffei* Ura5, with reported sequences of fungal OPRTase proteins by ClustalW analysis, revealed a high level of homology (data not shown). The sequence showed homology to OPRTase protein sequences from several fungi (71% to *Neosartorya fischeri* and 70% to *Aspergillus fumigatus*, *Aspergillus oryzae* and *Aspergillus terreus*), with the highest score of similarity to the OPRTase protein from *Aspergillus clavatus* (72%).

### 2. Differential gene expression during phase transition and stress-induced conditions

A shift in mycelium incubation temperature from 25°C to 37°C, which is required for the fungus to undergo a transition to yeast form, led to an increase in the *ura5* mRNA levels at 6 to 12 hr after shifting (Figure 2). However, the transcripts of this gene rapidly decreased paralleling with the differentiation of hyphae to yeast cells at 48 h. The expression pattern of the *ura5* gene was observed at normal homeostatic conditions (37°C) and during simulated human fever (39°C) or H<sub>2</sub>O<sub>2</sub>-treated yeast cells. At yeast phase, no difference of the expression levels was found in *ura5* gene when shifting the incubation temperatures from 37°C to 39°C, or when subjected to H<sub>2</sub>O<sub>2</sub> treatment. However, a substantial expression of *ura5* gene was found when shifting the incubation temperatures of the mycelium from 25°C to 39°C. A slight

upregulation of *ura5* gene was found when treating the cells with  $H_2O_2$ .

The investigation of the expression pattern of conidial *ura5* gene during macrophage infection revealed no difference of *ura5* gene expression between conidia incubated in cell-free medium and macrophage infection (Figure 3). However, the expression of *ura5* gene was upregulated after 4 hours of incubation both in conidia incubated in cell-free medium and during macrophage infection, when compared to the results of the 2-hour incubation.

## Discussion

Isolation of the *ura5* gene was successfully performed using degenerate primers. Gene characterization demonstrated that the number of nucleotides and amino acids for an open reading frame of *ura5* correspond to well-known fungal *URA5*s. Deduced amino acid sequences from the full-length *ura5* transcript displayed strong homology to OPRTase protein in other fungi, with the highest score of similarity to OPRTase protein from *Aspergillus clavatus* (72%). These results strongly suggest that isolated *ura5* gene is indeed OPRTase- encoding gene in *P. marneffei*. This gene could be further used as a homologous selectable marker for fungal transformation.

It has been reported that the *URA5* gene is required for virulence in *Histoplasma capsulatum* (Retallack *et al.*, 1999). Additionally, sequence tag (EST) analysis demonstrated the upregulation of the *ura5* gene during mycelium to yeast transition of *Paracoccidioides brasiliensis* (Bastos *et al.*, 2007) and the yeast cells of this fungus have been recovered from infected mouse liver (Costa *et al.*, 2007). In our experiment, the expression of *ura5*

in *P. marneffei* was observed during mold to yeast phase transition, which is thought to be the pathogenetic step of *P. marneffei* (Vanittanakom *et al.*, 2006), and during macrophage infection. The present study demonstrated that the expression of *ura5* gene was upregulated during the temperature upshift for inducing mycelium to yeast phase transition as that found in *P. brasiliensis*. Additionally, the expression of *ura5* was increased during conidia incubation in either cell-free medium or during macrophage infection for 4 hours, compared to the 2-hour incubation. Our findings indicate that upregulation of *ura5* expression is found at the temperature increase during mold-to-yeast phase transition and during conidia incubation at 37°C, both outside and inside macrophages.

In the study of *ura5* gene response to stress conditions, it was found that the expression of *ura5* was upregulated when mycelial cells encountered the heat shock and  $H_2O_2$ -treated conditions while the yeast cells showed no difference of *ura5* expression. This finding indicates that the *ura5* gene expresses in response to stress conditions by cell type-dependent as it was upregulated only in mycelium form.

In conclusion, *ura5* gene was isolated and characterized from *P. marneffei*. The expression of *ura5* gene was upregulated at the early hours of temperature-induced phase transition from mold to yeast, with a substantial upregulation in the stress conditions.

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