Molecular Cloning of the cDNA of Heat Shock Protein 88 Gene from the Entomopathogenic Fungus, *Paecilomyces tenuipes* Jocheon-1

Ya-qi Liu, Nam Sook Park, Yong Gyun Kim, Keun Ki Kim, Hyun Chul Park, Hong Joo Son, Chang Ho Hong, and Sang Mong Lee*

Department of Life Science & Environmental Biochemistry, College of Natural Resources and Life Science, Pusan National University, Miryang 627-706 South Korea

Abstract

The full-length heat shock protein 88 (HSP88) complementary DNA (cDNA) of *Paecilomyces tenuipes* Jocheon -1 was obtained by screening the *Paecilomyces tenuipes* (*P. tenuipes*) Jocheon-1 Uni-Zap cDNA library and performing 5' RACE polymerase chain reaction (PCR). The *P. tenuipes* Jocheon-1 HSP88 cDNA contained an open reading frame (ORF) of 2,139-basepair encoding 713 amino acid residues. The deduced amino acid sequence of the *P. tenuipes* Jocheon-1 HSP88 cDNA showed 77% identity to *Nectria haematococca* HSP88 and 45-76% identity to other fungal homologous HSP88s. Phylogenetic analysis and BLAST program analysis confirmed that the deduced amino acid sequences of the *P. tenuipes* Jocheon-1 HSP88 gene belonged to the ascomycetes group within the fungal clade. The *P. tenuipes* Jocheon-1 HSP88 also contained the conserved ATPase domain at the N-terminal region. The cDNA encoding *P. tenuipes* Jocheon-1 HSP88 was expressed as an 88 kilodalton (kDa) polypeptide in baculovirus-infected insect Sf9 cells. Under higher temperature conditions for the growth of the entomopathogenic fungus, mRNA expression of *P. tenuipes* Jocheon-1 HSP88 was quantified by real time PCR (qPCR). The results showed that heat shock stress induced a higher level of mRNA expression compared to normal growth conditions.

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Introduction

Heat shock proteins (HSPs) are a specific group of proteins that can be induced by heat shock (or stress) in a wide range of organisms including bacteria, plants, insects, mammals, and humans (Lemaux *et al.*, 1978; Yamamori *et al.*, 1978; Kelley and Schlesinger, 1978 ; Miller *et al.*, 1979 ; McAlister and Finkelstein, 1980 ; Johnston *et al.*, 1980 ; Manjunatha *et al.*,

2010 ; Routray *et al.*, 2013). As the name suggests, HSPs are induced in cells, tissues, or individuals exposed to sublethal heat shock. HSPs were first discovered in 1962 by Ritossa . It was reported that when *Drosophila* salivary gland cells were exposed to 37°C for 30 min then returned to their normal temperature of 25°C for recovery, a “puffing” of the corresponding genes was induced in the chromosomes of the recovering cells (Ritossa, 1962). In the 1970s, researchers found that this phenomenon was

*Corresponding author.

Sang Mong Lee

Department of Life Science & Environmental Biochemistry, College of Natural Resources and Life Science, Pusan National University, Miryang 627-706, South Korea

Tel: +82-55-350-5546 / FAX: +82-55-350-5549

E-mail: serilsm@pusan.ac.kr

accompanied by an increase in the expression of proteins with molecular masses of 26 and 70 kilodalton (kDa) (Tissieres *et al.*, 1974). These proteins were named “heat shock proteins”. Since these discoveries were made, a large number of proteins, referred to as HSPs, have been reported.

In addition to heat shock, the synthesis of HSPs can be induced by a variety of chemical and environmental stimuli. Exposure of cells to heavy metals (Levinson *et al.*, 1980), amino acid analogs (Kelley and Schlesinger, 1978; Li and Laszlo, 1985), glucose analogs (Pouyssegur *et al.*, 1977), Ca^{2+} increasing agents (Ding *et al.*, 1996), ischemia, sodium arsenite (Johnson *et al.*, 1980), microbial infections, nitric oxide, hormones, and antibiotics also induce the expression of HSPs. HSPs have been shown to perform multiple functions in cellular processes. Notably, HSPs function as molecular chaperones (Ellis, 1987) to facilitate proper synthesis and folding of proteins throughout the cell (Walter and Buchner, 2002; Martin and Ulrich, 1997). They also stabilize and assist in the trafficking of nascent peptides during normal growth (Hartl *et al.*, 1992). Under stressful conditions such as heat shock or hypoxia (Hammerer, 2001), increased expression of HSPs protects the cell by stabilizing unfolded or misfolded peptides and by allowing the cell time to repair or re-synthesize damaged proteins (Saibil, 2000). HSPs have been shown to participate in protein assembly (Welch, 1991), secretion (Wild *et al.*, 1992), and degradation as well as cellular homeostasis (Mallouk *et al.*, 1999), and the regulation of transcription factors and protein kinases (Sonya *et al.*, 2003). Additionally, HSPs have appeared to serve a protective role in cardiovascular health by aiding (Benjamin and McMillan, 1998) smooth muscle relaxation (McLemore *et al.*, 2005), preventing platelet aggregation, improving cardiac myocyte function, and preventing apoptosis after ischemic injury (Fan, 2005). Extracellular and membrane bound HSPs are involved in binding antigens and presenting them to the immune system (Nishikawa *et al.*, 2008); therefore, HSPs are useful as immunologic adjuvants in boosting the response to a vaccine (Bendz *et al.*, 2007). It has been speculated that HSPs may be useful for increasing the effectiveness of cancer vaccines (Binder, 2008).

Recent studies on HSPs have implicated their role in physiological, biochemical, molecular, biological, ecological, evolutionary, and clinical research. These studies have led to cloning and characterization of genes encoding HSPs in many organisms including the entomopathogenic fungus in our

present study. Until now, a great number of variable species of HSPs including small HSPs with molecular weights ranging from 15-40 kDa and many types of HSPs with molecular weights over 40 kDa have been studied; however, HSP88 is rarely investigated (Schlesinger, 1990; Sorensen *et al.*, 2003; Davis, 2004; Burnie *et al.*, 2006; Bendz *et al.*, 2007; Tsan and Gao, 2009; Manjunatha *et al.*, 2010; Shim *et al.*, 2012; Routray *et al.*, 2013). Although the amino acid sequence of the HSP88 has been reported from a fungal species (Plesofsky-Vig and Brambl, 1998), there are few reports regarding the full-length cDNA sequence. This is the first report, to our knowledge, about the HSP88 from *Paecilomyces tenuipes* Jocheon-1 (*P. tenuipes* Jocheon-1), an entomopathogenic fungus also known as the vegetable wasp, and plant worm. *P. tenuipes* Jocheon-1 is an ecotype of the corresponding species. Dongchoonghacho, a kind of silkworm mushroom produced from the entomopathogenic fungus of *P. tenuipes*, has been known as a mysterious ancient medicine in Asian countries, particularly in China, Korea, and Japan. This paper describes the molecular cloning of HSP88 cDNA from *P. tenuipes* Jocheon-1, the expression of cDNA, the deduced amino acid sequence of the protein, and the influence of the heat shock treatment on the corresponding mRNA expression levels.

Materials and Methods

Fungal strains

The entomopathogenic fungal strain, *P. tenuipes* Jocheon-1, was used in this study. This strain was collected from the mountainous region of Miryang, South Korea and was artificially cultured in egg yolk medium as described previously (Lee *et al.*, 2006).

Construction of the cDNA library and sequence analysis of expressed sequence tags (ESTs)

Total RNA was isolated from the fruiting body of *P. tenuipes* Jocheon-1 using the SV Total RNA Isolation System (Promega). mRNA was isolated using the oligotex mRNA midi kit (Qiagen, Korea Ltd). cDNA was synthesized using the Uni-ZAP cDNA synthesis kit (Stratagene) according to the manufacturer's

instruction. cDNA was ligated into the *EcoRI* and *XhoI* sites of the Uni-ZAP XR vector and packaged in the high efficiency system GigapackIII Gold packaging extract (Stratagene).

The Uni-ZAP XR lambda cDNA library clones were converted to the pBluescript SK(-) phagemid by *in vivo* excision as described by the manufacturer. *Escherichia coli* (*E. coli*) SOLR cells were infected with the phagemid, plated onto LB ampicillin agar plates, and incubated at 37°C overnight. Colonies appearing on the plate with the cDNA insert were selected for plasmid isolation. Plasmid DNA isolation was extracted using Accuprep plasmid mini extraction kit (Bioneer). Plasmids were then subjected to sequence analysis. *P. tenuipes* Jocheon-1 ESTs were compared with the BLASTX of the NCBI database to identify the homologous genes.

According to the *P. tenuipes* Jocheon-1 UniZAP cDNA library sequencing results, one clone with the insert of 1,444 basepairs (bp) showed higher similarity to the previously reported fungal HSP88. This clone contained the complete 3'-end with a poly(A) tail, but it was incomplete at the 5'-end. In order to obtain the full-length sequence of this predicted HSP88 cDNA, 5' RACE PCR was performed using the Marathon cDNA amplification kit (Clontech) according to the manufacturer's protocol. In order to guarantee the specificity of the PCR product, Nested PCR was performed using the following primers: Gene specific primer (GSP), nested gene specific primer (NGSP), adaptor primer 1 (AP1), and adaptor primer 2 (AP2) were supplied with the marathon cDNA amplification kit (Clontech). Amplifications were performed with a MyCycler Thermal Cycler (BioRad). The two specific primers were designed based on this partial sequence information. In the 5' RACE reaction, adaptor-ligated cDNA was generated from poly(A) RNA isolated from the fruiting body of *P. tenuipes* Jocheon-1. The first PCR amplification was carried out using lower GSP1 (5'-TGAATGTTAACACGGGTCTTAAGA-3') and AP1 (5'-CCATCCTAATACGACTCAC-TATAGGGC-3'). PCR cycling conditions were as follows: 33 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min followed by a terminal extension cycle at 72°C for 7 min. The second PCR amplification was carried out using upper GSP2 (5'-TGAATGTTAACACGGGTCTTAAGA-3') and AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3'). The PCR cycling conditions were the same as with the first PCR cycling conditions except

that the annealing temperature was 63°C. The PCR product was analyzed with a 1% agarose gel electrophoresis, and the band of approximately 1.4 kilobasepairs (kbp) was excised from the gel. The band was then purified with the AccuPrep Gel purification kit (Bioneer) and cloned to the pGEM-T Easy vector (Promega, USA) following the manufacturer's instruction. Blue-white screening of HIT competent DH5a cells was used to identify potential transformants. Positive clones were selected and sequenced with an Applied Biosystem 3730XI DNA Analyzer (USA). The 5' region sequencing result was combined with the poly(A) tail connected to the 3' region sequence to make the predicted full-length cDNA sequence.

Cloning and sequencing of the full-length *P. tenuipes* Jocheon-1 HSP88 cDNA

To isolate the full-length clone, the Marathon cDNA library of *P. tenuipes* Jocheon-1 was screened with the HSP forward primer (PtHSP-F 5'-AGCAACCATGAGTGTCGTCGGTG-3') and the HSP reverse primer (PtHSP-R 5'-CGTGATATCTATTGGAATCGGC-3'). These were designed based on the combined sequence information of the 5'-end sequence and the previously obtained partial sequence. Samples were run on a thermal cycle (BioRad) using PCR PreMix (Bioneer) under the following conditions: 35 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 2.5 min. The amplified fragment of approximately 2.1 kbp was purified and cloned to the pGEM-T Easy Vector that was then transformed to HIT competent DH5a cells (RH617, RBC). The plasmid was isolated using a plasmid mini extraction kit and sequenced with an Applied Biosystems 3730xl DNA Analyzer (USA).

Sequence analysis and phylogenetic tree construction

The amino acid sequences of other reported HSP88s were retrieved from the GenBank database provided by the NCBI. The sequences were aligned in clustal W (Thompson *et al.*, 1994). Molecular evolutionary genetics analysis (MEGA) software was used for statistical and phylogenetic analysis (Kumar *et al.*, 1994). The phylogenetic tree was constructed with the neighbor joining method (Saitou and Nei, 1987).

Construction of the baculovirus transfer vector

The 2,139-bp open reading frame (ORF) of HSP88 cDNA from *P. tenuipes* Jocheon-1 was subcloned between the *Sbf*I and *Bgl*II sites of pBacPAK9 (Clontech) to produce a baculovirus transfer vector pBacPAK9-PtHSP88. In the transfer vector, the *P. tenuipes* Jocheon-1 HSP88 cDNA was under the control of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV).

Cell culture and virus

Spodoptera frugiperda IPLB Sf21-AE (Vaughn *et al.*, 1977) clone9 (Sf9) cells were maintained at 27°C in TC100 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma). Cells were cultured as described by standard methods (O'Reilly *et al.*, 1992). Wild type AcNPV and recombinant AcNPV were propagated in Sf9 cells. The titer was expressed as plaque forming units (PFU) per mL (O'Reilly *et al.*, 1992).

Construction of the recombinant virus

Thirty-five milliliter cell culture dishes were seeded with $1.0\text{--}1.5 \times 10^6$ cells and incubated at 27°C for 1 h to allow cell attachment. One microgram of BacPAK6 viral DNA (Clontech), 5 µg of pBacPAK9-PtHSP88 in 20 mM HEPES buffer, and sterile water were mixed in a polystyrene tube for a total volume of 50 µL. Fifty µL of 100 µg/mL Lipofectin (Gibco Brl Life Technologies) were gently mixed with the DNA solution and the mixture was incubated at the room temperature for 30 min. The cells were washed twice with 2 mL serum free TC100 medium and fed with 1.5 mL serum free TC100 medium. The Lipofectin-DNA complexes were added in drops to the medium covering the cells while each dish was gently swirled. After incubation at 27°C for 5 h, TC100 medium containing antibiotics and 10% FBS was added to each dish and returned to incubation at 27°C. After 3 d, the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C until use. Recombinant AcNPV was plaque purified as described by O'Reilly *et al.* (1992).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sf9 insect cells were mock-infected, infected with wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV), or recombinant AcNPVs at a multiplicity of infection of 10^6 in 35 mm dishes. After incubation at 27°C for 3 d, cells were harvested. For SDS-PAGE of cell lysates, uninfected cells and virus infected Sf9 cells were washed twice with phosphate buffered saline (PBS), mixed with protein sample buffer, and boiled. Total cellular lysates were then subjected to 10% SDS-PAGE.

Stress treatment

The normal temperature for cultivation of *P. tenuipes* Jocheon-1 in the developmental stage of fruit body is approximately 20°C. In order to investigate the effects of heat shock treatments on the expression level of HSP88 mRNA in the early fruit body developmental stage, *P. tenuipes* Jocheon-1 were kept at 25°C and 30°C for different amounts of time, 8 h, 16 h, or 24 h.

Quantitative real time PCR (qPCR)

qPCR was used to measure the relative expression levels of HSP88 during the developmental stages of mycelium, early fruit body, middle fruit body, and late fruit body, respectively. qPCR has also previously been performed to analyze the effect of heat treatment on the relative expression level of HSP88 when *P. tenuipes* Jocheon-1 was in early fruit body development. One µg of total RNA was used for cDNA synthesis. cDNA was synthesized using the iScript cDNA synthesis kit (Biorad). qPCR was performed using an iQ5™ multicolor real time PCR detection system (BioRad) and sensimix SYBR kit (Bioline, according to the manufacturer's instructions.

Two HSP88 specific primers (RT-HSP-F ; 5'-GCCAGTGAAGAGGAGAAG-3' and RT-HSP-R; 5'-GCGATGTAGACACCCTTT-3') were used to amplify a PCR product of 101 bp. Two GAPDH primers (GAPDH-F; 5'-CACTCCTACACCGCTACC-3', GAPDH-R; 5'-GAATGACCTTGCCGACAG-3') were used to amplify a GAPDH gene fragment of 130 bp. GAPDH primers were based

on sequences from GenBank Accession number GU997101. GAPDH was used as an internal control for qPCR. The qPCR amplifications were carried out in total volume of 20 μ L containing 10 μ L of SYBR, 1 μ L of cDNA, 0.5 μ L of each forward and reverse primer (5 pmol final concentration), and 8 μ L of DEPC water.

Reactions were initiated with a denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 30 s, 72°C for 20 s, and a dissociation curve at 95°C for 60 s, 55°C for 60 s, and 60°C for 10 s. Two independent biological replicates were carried out. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. The comparative cycle threshold (Ct) method was used to analyze the expression level of HSP88.

Results

Cloning the full sequence of *P. tenuipes* Jocheon-1 HSP88 cDNA and data analysis

A cDNA library was constructed using fruiting bodies of *P. tenuipes* Jocheon-1. One thousand clones were randomly selected. Clones that were of low quality, too short (<700 bp), or had vector contamination were not included. Seven hundred and forty one ESTs were generated by a partial sequencing from the cDNA library. Of the 741 ESTs, 305 showed homology to genes identified and deposited in the NCBI database, 261 matched to unknown or unclassified functional genes, and 175 displayed similarity with an E-value that was lower than the adopted standard value of 10⁻⁵ in the process of functional annotation. According to the EST results of the cDNA library, one clone with an insert of 1,444 bp showed high homology to the previously reported HSP88 gene. The sequence contained the complete 3'-end with a poly(A) tail, but it was not complete at the 5'-end. Accordingly, the marathon 5' RACE PCR amplification method was used to determine the unknown 5'-end sequence as described previously. The PCR product was excised from the gel, purified with a gel extraction kit (Promega), and subcloned into the pGEM-T Easy vector for sequence analysis.

The DNA fragment of approximately 2.1 kbp that amplified using the primer pairs PtHSP-F and PtHSP-R was excised

and subcloned into the pGEM-T Easy vector for sequencing. The nucleotide and deduced amino acid sequences of HSP88 cDNA are represented in Fig. 1. The cDNA contained an ORF of 2,139-bp encoding 713 amino acid residues. From the nucleotide and deduced amino acid sequence of the cDNA, five sequence motifs named phosphate1, connect1, phosphate2, adenosine, and connect2, which involved in ATP binding and interdomain coupling (Buchberger *et al.*, 1994), have been identified (Fig. 1). Alignment of deduced amino acid sequences of *P. tenuipes* Jocheon-1 HSP88 cDNA with other fungal HSP88s is represented in Fig. 2. This data demonstrates that there are variable identities in the amino acid sequences of the homologous genes ranging from 45-77% (Fig. 2 and Table 1). Further comparison of the *P. tenuipes* Jocheon-1 HSP88 sequence with the ATPase domain of HSP70, the ATP-binding cell division protein FtsA of *E. coli* (Bork *et al.*, 1992), and the fungal HSP88 of *Neurospora crassa* (*N. crassa*) (Plesofsky-Vig and Brambl, 1998) revealed that *P. tenuipes* Jocheon-1 HSP88 also contains the conserved ATPase domain at the N-terminal region (Fig. 1).

A phylogenetic analysis using the deduced amino acid sequences of known fungal HSP88 genes revealed that it is divided into two separate clades, ascomycetes and basidiomycetes (Fig. 3). Within the ascomycetes fungal clade, *P. tenuipes* Jocheon-1 and *Nectria haematococca* (*N. haematococca*) formed a subgroup, *P. tenuipes* Jocheon-1 HSP88 showed the highest protein sequence identity (77%) to *N. haematococca*. The identity of *P. tenuipes* Jocheon-1 to other known HSP88s was 45-76% (Table1).

Expression of *P. tenuipes* Jocheon-1 HSP88 cDNA in baculovirus-infected insect cells

To assess the *P. tenuipes* Jocheon-1 HSP88 gene, the ORF of HSP88 cDNA, which was comprised of 2,139 nucleotides, was inserted into a baculovirus transfer vector. The transfer vector pBacPAK9-PtHSP88 was constructed by inserting *P. tenuipes* Jocheon-1 HSP88 cDNA under the control of the AcNPV polyhedrin promoter of pBacPAK9. In order to confirm the correct insertion of the HSP88 ORF fragment, the transfer vector pBacPAK9-PtHSP88 was double digested with restriction enzymes *Sbf*I and *Bgl*II. The baculovirus transfer vector was used to generate a recombinant virus expressing *P. tenuipes* Jocheon-1 HSP88. The recombinant AcNPV (AcNPV-PtHSP88) was

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-198 AAATCTTTCATCCCACCG
-180 TCTACCGTCTTATCCCCTCCCCCACCTCGATCCTCCAAACAAACCAACCCAACTTGCCT
-120 CCCCATATAGACTTCAGCGTCTTGGTTTCTGCATTAACTCGCGGCTGATTGGGGAAGAT
-60 TCTTTTCTGACCTCCCTCCCCCTTCCTATACACACATTACCTCATCACGCAGCAACC

+1 ATGAGTGTCTGTCGGTGTTCGATTTCGGTACCTCAAGACGGTCATTGCCGTCGCCAGAAAC
  1  M  S  V  V  G  V  D  F  G  T  L  K  T  V  I  A  V  A  R  N
                                P1
 61  CGCGGTGTCTGATGTGGTCACCAACGAGGTCTCCAACCGCTCTACTCCGTCTCTCGTCGGA
 21  R  G  V  D  V  V  T  N  E  V  S  N  R  S  T  P  S  L  V  G
                                P1
121  TTCGGTCCCAGGTCCCGTTATCTTGGCGAGACGGCCAAGACACAGGAAATCTCCAACCTC
 41  F  G  P  R  S  R  Y  L  G  E  T  A  K  T  Q  E  I  S  N  L

181  AAGAACACCGTCAACTGCCTTAAGCGCCTTGCCGGTCGCGCCTTTAACGACCCCGATGTC
 61  K  N  T  V  N  C  L  K  R  L  A  G  R  A  F  N  D  P  D  V

241  CAGATTGAGCAGCAGTTCATCACTGCTCCCTCGTTGACATCAACGGTCAGGTCGGTGCT
 81  Q  I  E  Q  Q  F  I  T  A  P  L  V  D  I  N  G  Q  V  G  A

301  GAGGTGAGCTACCTAGGCAAGCAGGAGAGATTTACTGCCACTCAGCTCGTCGGCATGTAC
101  E  V  S  Y  L  G  K  Q  E  R  F  T  A  T  Q  L  V  G  M  Y

361  CTCAGCAAGATCAAGCAGACCGCTGCCAACGAGCTCAAGCTCCCGTCTCCGACCTCTGC
121  L  S  K  I  K  Q  T  A  A  N  E  L  K  L  P  V  S  D  L  C

421  ATGAGCGTCCCGCCTTGGTTTACCGACGCGCAGCGCCGTGCCCTTCTCGATGCCTCTGAA
141  M  S  V  P  P  W  F  T  D  A  Q  R  R  A  L  L  D  A  S  E

481  CTCGCCGGCCTGAAGCTCCTCCGCCTCATCAATGACAACACTGCCGCCGTCTCGGCTGG
161  L  A  G  L  K  L  L  R  L  I  N  D  N  T  A  A  A  L  G  W
                                C1
541  GGCATTACCAAGCTCGACCTCCCTGCTACCGAGGAGCCCCCAAGCGTGTCTTCTTCATC
181  G  I  T  K  L  D  L  P  A  T  E  E  P  P  K  R  V  F  F  I
                                C1                                P2
601  GACATTGGCCACTCCAACCTACACCTGCTCCATTGTTGAGTTCAAGAAGGGCGAGCTAGCC
201  D  I  G  H  S  N  Y  T  C  S  I  V  E  F  K  K  G  E  L  A
                                P2
661  GTCAAGGCCACTGCTTGGGACCGCAACTTTGGTGGCCGTGACTTTGACAAGGCCCTCGTC
221  V  K  A  T  A  W  D  R  N  F  G  G  R  D  F  D  K  A  L  V

721  GACTACCTCGCCAAGGAGTTCAAGACCAAGTACAACGCCGACATCTACACCCATGGCCG
241  D  Y  L  A  K  E  F  K  T  K  Y  N  A  D  I  Y  T  H  G  R

781  GCCATGGCCCGTACCATTGCCGCCGCCGAGAAGTGCAAAAAGGTCTCTCCGCCAACCAG
261  A  M  A  R  T  I  A  A  A  E  K  C  K  K  V  L  S  A  N  Q

841  CAGTCCGCCGTCAACATTGAGTCTCTCATGAACGACATTGACGTTTCTGCCAATGTCACC
281  Q  S  A  V  N  I  E  S  L  M  N  D  I  D  V  S  A  N  V  T

901  CGCCAAGACTTTGAGGAGATGATCCAGCCGCTCCTTAACCGCACCTTTGAGCCCTCGAG
301  R  Q  D  F  E  E  M  I  Q  P  L  L  N  R  T  F  E  P  L  E

961  GCCGCACTCGCCAGGCCAAGCTCACCAAGGAAGACATCGATATTGTCGAGATTGTTGGC
321  A  A  L  A  Q  A  K  L  T  K  E  D  I  D  I  V  E  I  V  G
                                A

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Fig . 1. The nucleotide sequence, deduced amino acid sequence, and ATPase domain of the *P. tenuipes* Jocheon-1 HSP88 cDNA. The start codon, ATG, is boxed, and the termination codon is denoted with the asterisk. The poly(A) signal sequence is shown with the shadow mark. P1, phosphate1; P2, phosphate2; C1, connect1 (interdomain hinge); C2, connect2 (interdomain hinge); A, adenosine. This cDNA sequence has been provided with the accession number GU983958 from GenBank.

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1021 GCGCGCTCCCGTGTTCTCCTGCCCTCAAGGAGCGCCTCCAGTCCTTCTTTGGCAAGCCCTC
341 G G S R V P A L K E R L Q S F F G K P L
      A
1081 TCATACACCATGAACGCCGATGAGGCCATTGCTCGCGGCTGCGCCTTTAGCTGCGCCATT
361 S Y T M N A D E A I A R G C A F S C A I
      C2
1141 CTCTCGCCCGTATTCCGCGTCCGTGACTTTGCCGTTGAGGACATTGTCTAGCTACCCCTATC
381 L S P V F R V R D F A V Q D I V S Y P I
      C2
1201 GAGTTCACCTTGGGAGAAGGCTGCCGACATCCCTGACGAGGATACCAGCCTGACCGTCTTT
401 E F T W E K A A D I P D E D T S L T V F

1261 AACAAAGGCGGTCTTCTACCCCTCGACCAAGATCCTGACTTTCTACCGCAAGCAGCCCTAT
421 N K G G L L P S T K I L T F Y R K Q P Y

1321 GATCTCGAGGCCCGCTACGCCAAACCCGAGGACCTCCCCGAAAGCAGAATCCCTGGATC
441 D L E A R Y A K P E D L P G K Q N P W I

1381 GGCCGCTTCTCTGTCAAGGGTGTCAAACCTACTGAGACGGACGACTTTATGGTCTGCCGT
461 G R F S V K G V K P T E T D D F M V C R

1441 CTTAAGACCCGTGTTAACATTACGGCATCTTGAACGTTGAGACTGGCTACCACGTTGTCT
481 L K T R V N I H G I L N V E T G Y H V V

1501 GAGCAGGAGGTTGAGGAGGAGGTCAAGGAAGATGGCGACCCCGATGCCATGGACACTGAC
501 E Q E V E E E V K E D G D P D A M D T D

1561 AAGAAGGATGACGCCCCCAAGAAGACCCGCAAGGTCAAGAAGCAAGTTGCGCGCCGCGAC
521 K K D D A P K K T R K V K K Q V R A G D

1621 CTGCCCATATCCAGCGGTACCTCGTCTCTCGACGATGCCACCCGCATOGCTCTCGCCGAG
541 L P I S S G T S S L D D A T R I A L A E

1681 AAGGAGGCTGCTATGGTCATGGAGGACAAGCTCGTCGCGGACACTGACGAGAAGAAGAAC
561 K E A A M V M E D K L V A D T D E K K N

1741 GAGCTCGAGGCTTACATCTACGATCTCCGTTCCAACTGGAGGAGCAGTACGCTGACCTC
581 E L E A Y I Y D L R S K L E E Q Y A D L

1801 GCCAGTGAAGAGGAGAAGGAGAAGATCCGCGCAAGCTTGAGGCTTCCGAGGACTGGCTC
601 A S E E E K E K I R A K L E A S E D W L

1861 TACGATGAGGGTGAGGATGCCTCAAAGGGTGTCTACATCGCCAAGATTGACGAGATCCGC
621 Y D E G E D A S K G V Y I A K I D E I R

1921 GCCATGGCTGGCCCCGTCGTCAGCGCCACTTTGAGAAGGTCGAGGCTGAGCGCCAGGTC
641 A M A G P V V Q R H F E K V E A E R Q V

1981 ATCCAAGAGCGCTACGAGGCCGAGCAGGCTGCCAAAAGAGCCGCTGATGAAGAGGCTCGC
661 I Q E R Y E A E Q A A K R A A D E E A R

2041 AAGGCTGCTGAGGCTGAGAAGGCCGCCGAGGGCGGCGCCGACGCCGAGATGAAGGATGCC
681 K A A E A E K A A E G G A D A E M K D A

2101 GACGGCGCCCCGCGCAAGATGCCGCTGCCGATTCCAAATAGATATCACGAGTTATGAAG
701 D G A P A E D A A A D S K *

2161 AAGGAGGAGTAGGCAGGCAAGCACGTTGAGGTGTTGATTGCAAAATGGCATTTTACTCTCA
2221 CGAAAGGCCAGAAGATGGAAGAAAGGCAGAAAGAGAAAAGCAACCGAGTCCTTTTGA
2281 TTTACGAAGAAGGCTCTGGTTTTTTTTTTTATTGGGACGCAATATAAACAAAAGAGGCA
2341 TGAGGAAAATGGGATATTCTGTTTCCTGTTTTTATAATGTTTTAGAAATGACAAATGAAA
2401 CGCTTTATAGAATGCGAANAANAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
2461 AAAAAAAAAAAAA

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Fig. 1. Continued.

<i>P. tenuipes</i> Jocheon1	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>N. haematococca</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>G. graminicola</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>N. crassa</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>M. oryzae</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>B. fuckeliana</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>S. sclerotiorum</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>A. dermatitidis</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>T. stipitatus</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>P. marneffeii</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>A. benhamiae</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>Cn var. neoformans</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>L. bicolor</i>	MSVVGVDGGLNIVIAVARNRGVIVITNEVSNRSTPSIVGFGPKSRYLGEAAKTQEISNLKNIVGSLKRLAGRAEFCPIV	80
<i>P. tenuipes</i> Jocheon1	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>N. haematococca</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>G. graminicola</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>N. crassa</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>M. oryzae</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>B. fuckeliana</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>S. sclerotiorum</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>A. dermatitidis</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>T. stipitatus</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>P. marneffeii</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>A. benhamiae</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>Cn var. neoformans</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>L. bicolor</i>	QTECCFIITAFIVLVNGCVGAEVSYLGGKKEFTATQIVAMYLKIKQTATAEELKLEVSDDMEVEFWFTDQRRALIDAA	159
<i>P. tenuipes</i> Jocheon1	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>N. haematococca</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>G. graminicola</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>N. crassa</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>M. oryzae</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>B. fuckeliana</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>S. sclerotiorum</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>A. dermatitidis</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>T. stipitatus</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>P. marneffeii</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>A. benhamiae</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>Cn var. neoformans</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>L. bicolor</i>	EIAGLKLRLINDITAAALGGITKLDLFEPEERFVAFVDIGHSNYTSIVVEFKAGELVKAFAKDRHFGGRDFDKAL	239
<i>P. tenuipes</i> Jocheon1	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>N. haematococca</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>G. graminicola</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>N. crassa</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>M. oryzae</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>B. fuckeliana</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>S. sclerotiorum</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>A. dermatitidis</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>T. stipitatus</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>P. marneffeii</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>A. benhamiae</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>Cn var. neoformans</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>L. bicolor</i>	VDHRAKEFFKAYKIDITSNPKANWVVAEAAEKLKILSANACAPLNIESIMNDIVSAMITRCEFEAMVEFLLAVFVFL	319
<i>P. tenuipes</i> Jocheon1	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>N. haematococca</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>G. graminicola</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>N. crassa</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>M. oryzae</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>B. fuckeliana</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>S. sclerotiorum</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>A. dermatitidis</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>T. stipitatus</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>P. marneffeii</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>A. benhamiae</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>Cn var. neoformans</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398
<i>L. bicolor</i>	EQALAEAKLTKEEDIDITIEVGGGSEFVPAKKEFIQEFFG-KPLSFTLNQDEAFARGCAFSFCAILSEVFFVRDFEFAHICIVNA	398

Fig. 2. Alignment of the amino acid sequences of *P. tenuipes* Jocheon-1 HSP88 with other known fungal HSP88s. GenBank accession numbers are: *P. tenuipes* Jocheon-1 (GU983958), *G. graminicola* (EFQ27583), *N. haematococca* (EEU34379), *M. oryzae* (EDJ96308), *N. crassa* (EDN96960), *S. Sclerotio* (EEP78775), *B. fuckeliana* (EDN30753), *T. stipitatu* (EED21621), *P. marneffeii* (EEA25736), *A. dermatitidis* (EEQ88845), *A. benhamiae* (EDN02919), *Cn var. neoformans* (AAW46676), and *L. bicolor* (EDR11689).

[illegible]

Table 1. Pairwise identities and similarities of the amino acid sequences of *P. tenuipes* Jocheon-1 HSP88 and other known HSP88 genes

	Percent Identity (%)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>P. tenuipes</i> Jocheon-1		77	76	71	71	69	69	65	67	67	64	46	45
<i>N. haematococca</i>	87		78	71	73	69	69	65	66	67	65	47	45
<i>G. graminicola</i>	88	87		75	77	74	74	67	67	69	66	48	46
<i>N. crassa</i>	85	84	88		76	71	71	66	68	67	66	49	47
<i>M. oryzae</i>	85	84	89	87		71	72	66	67	69	67	48	47
<i>B. fuckeliana</i>	85	83	87	86	86		96	70	68	70	69	50	49
<i>S. sclerotiorum</i>	84	82	86	86	85	98		70	69	70	69	50	50
<i>A. dermatitidis</i>	82	80	82	83	82	83	83		74	73	76	49	48
<i>T. stipitatus</i>	83	82	84	84	83	84	84	87		91	72	49	47
<i>P. mameffei</i>	83	81	83	83	84	84	84	87	96		74	50	48
<i>A. benhamiae</i>	80	79	81	81	82	84	83	86	85	87		49	48
<i>Cnvar. neoformans</i>	63	65	66	66	67	66	66	65	67	66	65		64
<i>L. bicolor</i>	64	65	65	65	67	67	68	66	66	66	66	79	

	Percent Similarity (%)												
<i>P. tenuipes</i> Jocheon-1		77	76	71	71	69	69	65	67	67	64	46	45
<i>N. haematococca</i>	87		78	71	73	69	69	65	66	67	65	47	45
<i>G. graminicola</i>	88	87		75	77	74	74	67	67	69	66	48	46
<i>N. crassa</i>	85	84	88		76	71	71	66	68	67	66	49	47
<i>M. oryzae</i>	85	84	89	87		71	72	66	67	69	67	48	47
<i>B. fuckeliana</i>	85	83	87	86	86		96	70	68	70	69	50	49
<i>S. sclerotiorum</i>	84	82	86	86	85	98		70	69	70	69	50	50
<i>A. dermatitidis</i>	82	80	82	83	82	83	83		74	73	76	49	48
<i>T. stipitatus</i>	83	82	84	84	83	84	84	87		91	72	49	47
<i>P. mameffei</i>	83	81	83	83	84	84	84	87	96		74	50	48
<i>A. benhamiae</i>	80	79	81	81	82	84	83	86	85	87		49	48
<i>Cnvar. neoformans</i>	63	65	66	66	67	66	66	65	67	66	65		64
<i>L. bicolor</i>	64	65	65	65	67	67	68	66	66	66	66	79

Fig. 3. Phylogenetic tree of aligned amino acid sequences of the *P. tenuipes* Jocheon-1 HSP88 and the other known fungal HSP88 sequences. The accession numbers of the sequences in the GenBank are as follows: *P. tenuipes* Jocheon-1 (GU983958), *N. hamematococca* (EEU34379), *G. graminicola* (EFQ27583), *N. crassa* (AAC23862), *M. oryzae* (EDJ96308), *B. fuckeliana* (EDN30735), *S. sclerotiorum* (EDN96960), *A. dermatitidis* (EEQ88845), *A. benhamiae* (EFQ98775), *T. stipitatus* (EED21621), *P. marneffei* (EEA25763), *Cn var. neoformans* (AAW46676), and *L. bicolor* (EDR11689).

produced in insect Sf9 cells by co-transfection with wild type AcNPV DNA and the transfer vector. To examine expression, the protein synthesized in Sf9 cells infected with the recombinant

Fig. 4. Expression of the *P. tenuipes* Jocheon-1 HSP88 cDNA in recombinant baculovirus-infected insect Sf9 cells. Lane1, size marker; Lane2, Sf9 cells that were mock-infected; Lane3, Sf9 cells that were infected with wild type AcNPV; and Lane4, Sf9 cells that infected with AcNPV recombinated with PtHSP88 cDNA. The total cellular lysates were subjected to 10% SDS-PAGE. The proteins on each lane were stained with Coomassie Brilliant Blue R250.

virus was analyzed by SDS-PAGE. The recombinant *P. tenuipes* Jocheon-1 HSP88 expressed by the *P. tenuipes* Jocheon-1

HSP88 cDNA was approximately 88 kDa as determined by the gel from the cells infected with the recombinant virus but was not detected from the cells infected with the wild type AcNPV or mock-infected cells (Fig. 4).

Expression profile of *P. tenuipes* Jocheon-1 HSP88 during different developmental stages under the normal temperature growth conditions

The relative expression level of *P. tenuipes* Jocheon-1 HSP88 mRNA during the four different developmental stages, mycelium, early fruit body, middle fruit body, and late fruit body, was detected by qPCR. The mycelium stage was used as the control for comparative purposes in normal growth conditions. The highest expression was observed in early fruit body development with expression approximately 2.5 fold higher than

that in the mycelium stage and 3.6 fold higher than that in late fruit stage (Fig. 5A).

Heat induced expression of *P. tenuipes* Jocheon-1 HSP88 cDNA

To ascertain the potential of heat induced expression of *P. tenuipes* Jocheon-1 HSP88 cDNA, the fruit body of *P. tenuipes* Jocheon-1 was subjected to heat shock at various temperatures and times. The relative expression level of the *P. tenuipes* Jocheon-1 HSP88 gene after heat treatment is shown in Figure 5B. During the heat shock treatment of 25°C, the expression level was highest at 16 h, which was 3.36 fold higher than that of the control at the starting point (0 h at 20°C in the fruit body). During the heat shock treatment at 30°C, the expression level of *P. tenuipes* Jocheon-1 HSP88 cDNA reached its highest at 16 h, which was 2.55 fold higher than that of the control at the starting point. After 24 h, expression had decreased to levels similar to the control at 24 h.

Fig. 5. Relative expression level of *P. tenuipes* Jocheon-1 HSP88 gene by real-time PCR. (A) Relative expression level of *P. tenuipes* Jocheon-1 HSP88 gene during different developing stages, (B) Relative expression level of *P. tenuipes* Jocheon-1 HSP88 after heat shock treatment.

Discussion

In this study, the full-length cDNA sequence of HSP88 was cloned from *P. tenuipes* Jocheon-1. The full-length cDNA of *P. tenuipes* Jocheon-1 is 2,139 bp and encodes a polypeptide of 713 amino acids. We had previously reported the genomic structure of the HSP88 genes in four entomopathogenic fungi, *P. tenuipes* Jocheon-1, *P. tenuipes*, *Cordyceps militaris* (*C. militaris*), and *Cordyceps pruinosa* (*C. pruinosa*), which are industrialized in Korea (Liu *et al.*, 2012). Among them the three entomopathogenic fungal strains, *P. tenuipes* Jocheon-1, *P. tenuipes*, and *C. militaris* showed identical genomic structure, namely 5 introns and 6 exons with 13, 62, 1,438, 306, and 288 nucleotides encoding 713 amino acid residues. *C. pruinosa* revealed a different species because it contained 4 introns and 5 exons with the length of 13, 62, 32, 1,744, and 288 nucleotides encoding 713 amino acid residues (Liu *et al.*, 2012). It is very interesting that almost same number of deduced amino acid residues for HSP88 in even different genera or species was identified. Pairwise comparison of *P. tenuipes* Jocheon-1 HSP88 with those of other fungal strains revealed a wide range of identity ranging from 45-77%. These results demonstrate less

identity than in the fruiting body forming insect mushroom species studied previously in 2012 (Liu *et al.*) This reveals that the HSP genes compared in this study are homologous (even variable in similarity of identity) to the ancestral HSP gene, but there are many mutations in the corresponding sequences that have occurred through long periods of evolutionary time.

The ATPase domains of HSP70, actin, and hexokinase have a common three-dimensional structure (Flaherty *et al.*, 1991). Five sequence motifs named phosphate1, connect1, phosphate2, adenosine, and connect2, which are involved in ATP binding and interdomain coupling (Buchberger *et al.*, 1994), have been identified within these structurally similar domains (Bork *et al.*, 1992). In this study, we found that these motifs are also present in the HSP88 of *P. tenuipes* Jocheon-1 and other fungal HSP88s, such as the HSP88 of *N. crassa* (Plesofsky-Vig and Brambl, 1998). Analysis of the amino acid sequence of this gene revealed a considerable identity with the fungal HSP88s of *N. haematococca*, *N. graminicola*, and *N. crassa*. Sequence conservation was highest at the N-terminus and decreased towards the C-terminus. The cDNA encoding *P. tenuipes* Jocheon-1 HSP88 was expressed as an 88-kDa polypeptide in baculovirus-infected insect Sf9 cells. Future research will continue on the construction of antibodies against HSP88 expressed from the recombinant baculovirus infected with PtHSP88 cDNA and identification of the protein with western blot analysis.

qPCR was performed to detect the relative expression level of *P. tenuipes* Jocheon-1 HSP88 during the developing stages of mycelium, early fruit body, middle fruit body, and late fruit body, respectively. Results showed that *P. tenuipes* Jocheon-1 HSP88 has the highest relative expression level during the early fruit body developmental stage. We conducted our experiments at 35°C, but sampling was difficult because of mycelial death at the treated temperature. In organisms that can grow over a broad range of temperatures, the maximum response is usually achieved at about 10°C above the optimum growth temperature. In organisms that grow over a more restricted range, the maximum response occurs at about 5°C above the optimum growth temperature. (Lindquist, 1986). The optimum temperature for the growth of *P. tenuipes* Jocheon-1 during fruit body development is approximately 20°C. Heat shock treatments at 25°C and 30°C have been performed and qPCR has been used to analyze the relative expression level of HSPs after heat shock.

Results demonstrated that the relative expression level of *P. tenuipes* Jocheon-1 HSP88 is greatest at 25°C after 16 h, which is approximately 3.5 times higher than that of control. The relative expression level of *P. tenuipes* Jocheon-1 HSP88 also increased during heat shock treatment at 30°C and reached its highest level after 16 h, and then levels reduced similar to those in the control group after 24 h. These results indicate that HSP88 production is probably dependent on both cellular differentiation and heat stress. The results obtained in this research will be useful to further investigate the molecular, biological, physiological, and developmental roles of the HSP88 in entomopathogenic fungi, which are very important species in the industrialization of insect mushrooms.

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