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Identification and characteristic analysis of the heat shock protein 70 of *Hypena tristalis* (Lepidoptera : Noctuidae)

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ABSTRACT

The experiment began in June 2011, and completed in December 2012 in the insects laboratory of the North-east Agricultural University, Harbin, China. The *Hypena tristalis* (*Htri*) was collected in the North-east Agricultural University Xiang Fang farm. For the purposes of research on the expression of heat shock protein 70 (HSP 70) gene of *H. tristalis* treated by heat stress, with changes in expression level in different induction times, RT-PCR technology was adopted for cloning to obtain a 1905 bp HSP 70 fragment of *H. tristalis* (GenBank accession number JQ316541), 635 amino acid residues were encoded, the predicted molecular weight was about 69.6 kD, and the isoelectric point was 5.36. The online bioinformatics prediction and analysis showed that the homology of the nucleotide sequence of *H. tristalis* HSP 70 with that of *Helicoverpa armigera* (accession number HM593518) was the highest one, which was 96%; the similarity was more than 70% while comparing with other insects. A 69.6 kD heterologous protein was detected in the HSP 70 prokaryotic expression (pET/*Htri*), and the protein was branded by Western blot detection. These results provide basic data for further research on the function of HSP 70 protein and for the pest control by thermal stress.

Key words : Gene cloning, heat shock protein 70, *Hypena tristalis*, sequence analysis

INTRODUCTION

Heat shock protein (HSP) is a category of highly conserved proteins, which are commonly found in prokaryotes and eukaryotes. They are produced when organisms under heat shock or stimulation by pathogens or other physical-chemical factors (Xia *et al.*, 2013). These proteins can be divided by molecular weight into the small molecular weight HSP family (small HSP), the HSP 60 family, HSP 70 family, HSP 90 family and HSP 110 family (Li *et al.*, 2009), among which the HSP 70 family is the most conservative and important one (Tang *et al.*, 2011).

Usually HSP 70 is considered as molecular chaperones (Wang and Jiang, 2010), which are capable of facilitating the folding of newly produced peptide chains and assisting in the transport of proteins between cells. HSP 70 is useful to maintain the stability of the internal environment of the insect cells, and accelerate the repair of damaged cells (Wang and Lei, 2005). The heat shock protein is expressed that is one of the most important effects of organisms adapting to changes in ambient temperature at molecular level.

Hypena tristalis belongs to Lepidoptera : Noctuidae, and it is an important defoliator in soybean fields. According to the report (Liu and Zhao, 2010), the emergence size of *H. tristalis* in the north-east of China has an upward trend in recent years, and *H. tristalis* has risen from a minor pest to a primary one and has a certain level of outbreaks. Currently, reports concerning *H. tristalis* are limited to survey on occurrence pattern of the pest, and few research on control of *H. tristalis* specifically in terms of HSP70 proteins has been carried out at home or abroad. In this article, HSP 70 gene of *H. tristalis* was obtained by cloning with RT-PCR method, and expressed HSP 70 recombinant proteins of *H. tristalis*, establishing a certain level of scientific foundation for researching the function of HSP 70 proteins after treating heat stress on *H. tristalis*, and for controlling pests by thermal control technology.

MATERIALS AND METHODS

The experiment began in June 2011, and completed in December 2012 in the insects laboratory of the Northeast Agricultural University, Harbin, China.

Test Insect

The *H. tristalis* was collected from the Xiang Fang Farm which is the experiment and practice base of the North-east Agricultural University. The larvaes were bred with natural feed in the insects lab of the North-east Agricultural University.

Culture and Vector

The pMD18-T vector kit was purchased from TaKaRa company. The *Escherichia coli* DH5α, the bacterial strain BL 21 (DE 3) and the plasmid pET21-b were all storage in the lab for this experiment.

Main Reagents and Tools

The total RNA isolation reagent TRIzol was purchased from Invitrogen company. The T_4 DNA Ligase, 3' RACE and 5' RACE Kit were purchased from TaKaRa company. The restriction enzymes *Not* I and *Xho* I were purchased from NEB company; the standard protein marker from Bio Rad company; the His-Tag Antibody and the HRP-labelled goat anti-mouse lgG from Biyuntian company. All other related reagents were domestic analytical reagents purchased from China.

Total RNA Isolation

After 30 min pretreatment of *H. tristalis* larva in a 40°C calorstat, the total RNA of *H. tristalis* larva was isolated with the TRIzol reagent of Invitrogen company in accordance with the instructions.

Primer Design

The primer for HSP 70 conservative domain was designed and synthesized according to the nucleotide sequence of HSP 70 genes from several kinds of Noctuidae insects recorded in GenBank. According to the instructions for the 3' RACE and 5' RACE Kits, the nested PCR primer was designed with the sequence obtained, which was in the conservative domain of *H. tristalis*. Three sequences were assembled and the primer for prokaryotic expression was designed.

For the convenience of cloning the target gene to the expression vector, the *Not* I and *Xho* I restriction enzyme cutting sites (underlined) were designed separately in the upstream and downstream of the expression primer.

RT-PCR Amplification

The RT-PCR amplification was done according to the instructions of RT-PCR kit (Table 1).

Cloning and Identification of PCR Products

1% agarose gel electrophoresis was used for detection after PCR amplification was performed on the target fragment. The PCR purified products were cloned to the pMD18-T vector, and then were transformed into *Escherichia coli* DH5α, plasmid was analysed by restriction enzyme digestion.

Sequencing, prediction and analysis : Samples of positive clones with a successful

Table 1. The code sequence and length of the primers

Code	Primer sequence	Length
Upstream of conservative domain	5'-GACATGAAGCACTGGCCCTTC-3'	27
Downstream of conservative domain	5'-TCGCTCGCTCGCCCTCGTACA-3'	27
3' RACE Outer	5'-ACTTCTTCTGTGGGAAGAAACTG-3'	23
3' RACE Inner	5'-AAGATTCCGTGCAAACAGTCTCAGACATTCAC-3'	32
5' RACE Outer	5'-GCTGGTACAGTGACAACCGCATC-3'	23
5' RACE Inner	5'-CGGAGATGACTTTGAAGGGCCAGTGCTTCATGTC-3'	34
Upstream of expression	5'-AAGGAAAAAAGCGGCCGCAATGGCAGCAACTAAAGCACCC-3'	40
Downstream of expression	5'-CCGCTCGAGGTCGACTTCTTCAACAGTTGGTCC-3'	33

RT-PCR Amplification : According to the instructions of RT-PCR kit.

enzyme cutting identification were sent to Shanghai Bioengineering Company for DNA sequencing, and the genes obtained were translated into amino acid sequences, and prediction and analysis were carried out with online bioinformatic tools. The isoelectric point, molecular weight, signal peptide and other information of the amino acid were predicted through http://www.expasy.ch/.

Construction of the recombinant expression plasmid : T4 ligase was used to mix the recombinant pMD18/*Htri*HSP70 (after double enzyme digestion and purification with *Not* I and *Xho* I) and the pET21-b vector (with the same double enzyme digestion) together at the mixed molar ratio of 3 : 1. The connection conditions were at 4°C over night, transformation into *E. coli* DH5α, and thermostatic cultivation at 37°C for 16 h. Single colonies were taken to isolate plasmids for *Not* I and *Xho* I double enzyme digestion, and 1% agarose gel electrophoresis was used for detection.

Induced expression : Single colonies were selected and activated in 5 ml LB liquid culture medium containing ampicillin. They were cultivated in a 200 rpm shaker at 37°C for 2 h. When the OD 600 value reached 0.3 or so, IPTG with a final concentration of 0.2 mMol/l was added for induction culture at 37°C. Bacteria solution induced for 0, 2 and 4 h were selected, respectively, as gradient for SDS-PAGE detection, and were transferred to BL 21 bacteria solution when compared as empty plasmid.

SDS-PAGE and western blot analysis : SDS-PAGE electrophoresis detection was performed to select the most appropriate application volume, and detection was performed again after purification of interest protein. The proteins on the SDS-PAGE gel were transferred to a PVDF membrane, and then they were set on ice over night with blocking buffer (TBST+5% ski mMed milk powder). Incubation with His-Tag Antibody 1:1000 at room temperature for 1 h; 3 times of TBST wash; reaction with HRP-labelled goat anti-mouse lgG 1:1000 for 1 h; three times of TBST wash and chemical colour reaction with DAB kit at last.

RESULTS AND DISCUSSION

Amplification and Cloning of HSP 70 Gene from *Hypena tristalis*

Obtaining of three gene sequences : PCR amplification products were obtained which were the conservative domain sequence fragments of 1096 bp, the fragments of 1065 and 440 bp, respectively, by 3' RACE and 5' RACE. Finally, the three sequences were assembled with AlignX software to predict the full length fragment of HSP 70 gene of *H. tristalis*.

Obtaining of gene full length : The total cDNA of *H. tristalis* larva after heat shock was used as template, and PCR amplification was performed with full length primers, and obtained specific bands of 2200 bp or so (Fig. 1). The target fragments were connected to the pMD18-T vector with T_4 ligase, and they were transformed into DH5α, and then identied the cloning results.

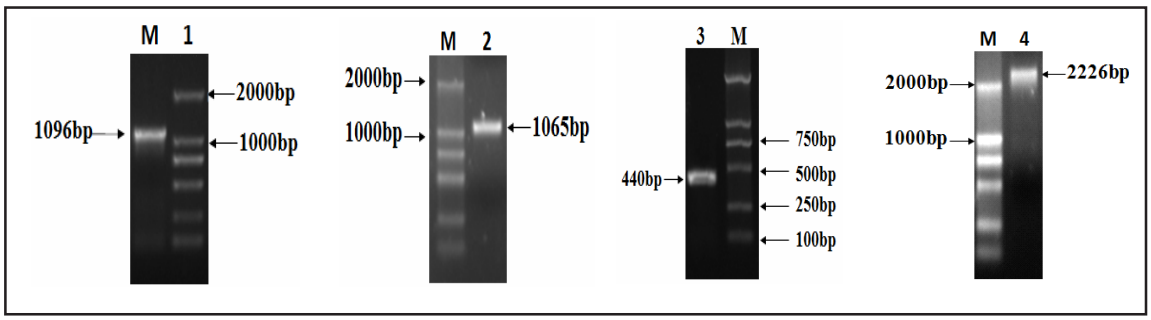

Fig. 1. PCR product of HSP 70 protein gene of *Hypena tristalis* (M : Molecular weight marker DL2000; 1 : Conservative region PCR products of *Hypena tristalis*; 2 : 3' RACE PCR products of *Hypena tristalis*; 3 : 5' RACE PCR products of *Hypena tristalis*; 4 : HSP 70 full-length cDNA PCR products of *Hypena tristalis).*

Sequencing and Analysis

The sequencing results of HSP 70 gene of *H. tristalis* showed that the full length was 2226 bp, containing an ORF of 1908 bp, and encoding 635 amino acid residues and presumable molecular weight of about 69.6 kD. This gene had been accessioned by GenBank (Accession Nunber : JQ316541). It was predicted that no signal peptide was present at N-terminus with an isoelectric point of 5.36, containing 5 N-glycosylation sites : NRTT (37~40 residues), NNTI (66~69 residues), NDSQ (153~156 residues), NLSI (363~366 residues) and NVSA (490~493 residues). Using protein analysis software for analysis, the protein had three signature sequences of the HSP 70 family : IDLGTTYS (11~18 residues) IPDLGGGTFDVSVL (199~212 residues) and VVLVGGSTRIPKIOS (337~351 residues). It had three eukaryotic cell motifs : the ATP-GTP binding sites AEAYLGTT (133~140 residues), non-organellar consensus motif RARFEELGPTVEEVD (302~308 residues) and cytoplasmic HSP 70 functional motif (629~635 residues).

According to the comparison results, the amino acid sequences of the *H. tristalis* HSP 70 had the highest similarity with the HSP 70 genes of *Helicoverpa armigera* (HM593518), *Mamestra brassicae* (AB251895), *Helicoverpa zea* (GQ389711), *Plutella xylostella* (HM212645), *Spodoptera exigua* (FJ754276), *Manduca sexta* (FJ754276), *Drosophila persimilis* (XM_002013420), and *Agrotis ipsilon* (JF809817), with the conformance of 96, 96, 95, 88, 86, 84, 84 and 77%, respectively. It was concluded from comparison and analysis of sequences that the N-terminus of HSP 70 amino acid of several insects were more conserved than the C-terminus, but the last fourth amino acid "EEVD" was highly conserved. The end of C-terminus contained the sequence for cellular localization, located in the sequence on the cytoplasm was "EEVD" (Yang *et al.*, 2009).

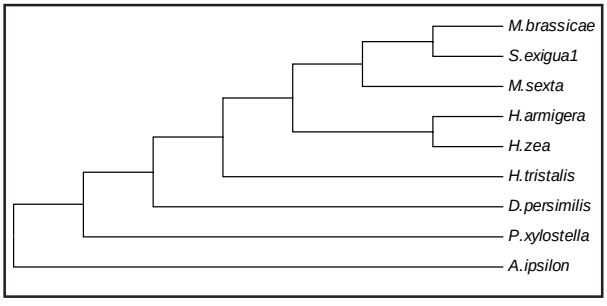

Fig. 2. Phylogenetic tree based on amino acid sequences of HSP 70.

The evolutionary systems of HSP 70 amino acid sequences of nine different species were analyzed by using ClustalX software. The results showed that (Fig. 2), *H. tristalis* had closer genetic relationship with *Mamestra brassicae*, *Spodoptera exigua*, *Helicoverpa armigera* and *Helicoverpa zea* (Lepidoptera : Noctuidae) and the *Manduca sexta* (Sphingidae), followed by *Drosophila persimilis* (Diptera) and farther relationship with *Plutella xylostella* (Lepidoptera : Plutellidae) and *Agrotis ipsilon* (Noctuidae). *H. tristalis* had the closest genetic relationship with amino acid sequences of most of the HSP 70 genes belonging to Noctuidae, and this was the same as other taxonomic status.

Construction of Prokaryotic Expression Vector for HSP 70 Gene from *H. tristalis*

The fragment of 1905 bp in size was obtained by recombinant cloning pMD18/*Htri*HSP70 plasmid. It was tested by extracting double enzyme digested plasmid, as shown in Fig. 3, indicating that pET/*Htri*HSP 70 expression plasmids were constructed successfully.

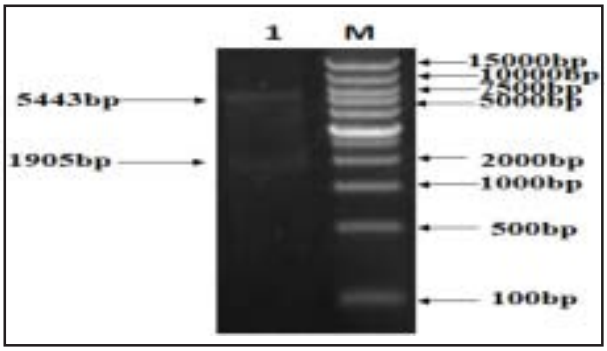

Fig. 3. Identification of positive clones by enzymatic digest (M : DNA marker DL2000; 1 : p pMD18/*Htri*HSP 70 was digested by *Not* I and *Xho* I).

Expression and Determination of HSP 70 Gene of *H. tristalis* in *E. coli*

The specific protein band of about 70 kD was obtained by inducing pET/*Htri*HSP70 plasmid in BL 21 with IPTG, while no corresponding specific band was found in the same pET21-b plasmid induced by IPTG (Fig.

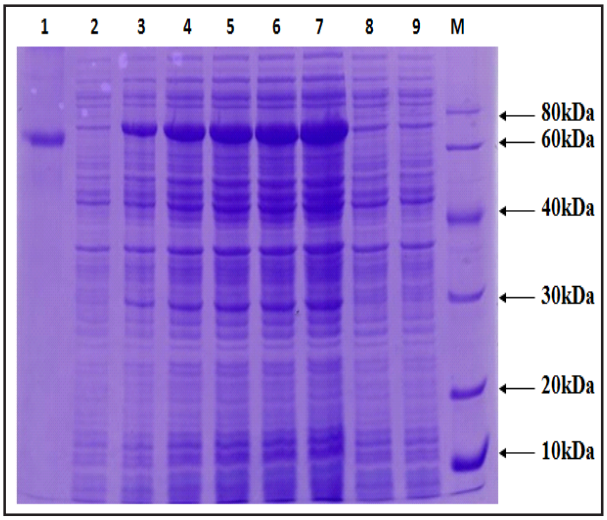

Fig. 4. SDS-PAGE analysis of the pET/*Htri*HSP 70 protein expressed products (M : Protein molecular weight marker; 1 : BSA; 2, 8, 9 : Expressed products of pET21-b; 3-7 : Expressed products of pET/*Htri*HSP 70 for 2, 3, 4, 5 and 6 µl loading quantity of protein samples.

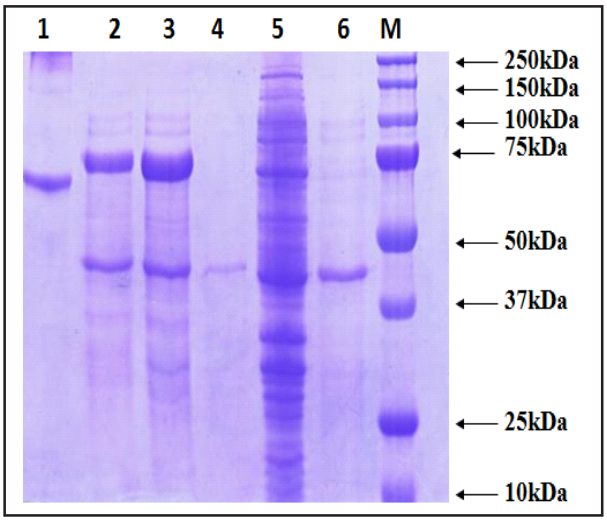

Fig. 5. SDS-PAGE analysis of the purified pET/*Htri*HSP 70 protein expressed products (M : Protein molecular weight marker; 1 : BSA; 2, 3, 4 : Expressed products of purified pET/*Htri*HSP 70 protein for 2, 4 and 0 h by IPTG induction; 5 : Expressed products of pET21-b and 6 : Expressed products of purified pET21-b protein).

4), indicating that the expression product was a fusion of HSP 70 target protein with 69.6 kD, in line with the characteristics of the HSP 70 protein family. The protein was purified and re-analyzed by SDS-PAGE, which was induced with IPTG for time gradient of 0, 2 and 4 h, yielding the results (Fig. 5). The expression of IPTG induction at 0 h was zero, and the expression was started from 2 h and the expression level was significantly lower than that of 4 h, indicating that 4 h was the optimal induction time. Western blot analysis was made to expression products. An apparent hybrid zone appeared at 69.6 kD (Fig. 6), which was fully consistent with the molecular size of the target protein, indicating that recombinant plasmids could be stably expressed in BL 21 (DE 3).

The amino acid sequence analysis showed that the gene contained three signature sequences of the HSP 70 family (Xie, 2009). The sequence alignment results showed that the HSP 70 gene sequences in different organisms had high homology, which proved that the HSP 70 gene was highly conserved between species and could be used as an indicator to measure the degree of evolution of species.

The results showed that no HSP 70 protein expression was found when the pET/*Htri*HSP70 plasmid in BL 21 was induced with IPTG for 0 h, while an apparent expression appeared at 2 and 4 h, and the expression level at 4 h was higher than at 2 h. Western blot analysis results were identical, so the HSP 70 protein was an induced protein.

Studies have shown that the HSP 70 protein in insects has the highest expression level at 40°C. In order to study the *H. tristalis*

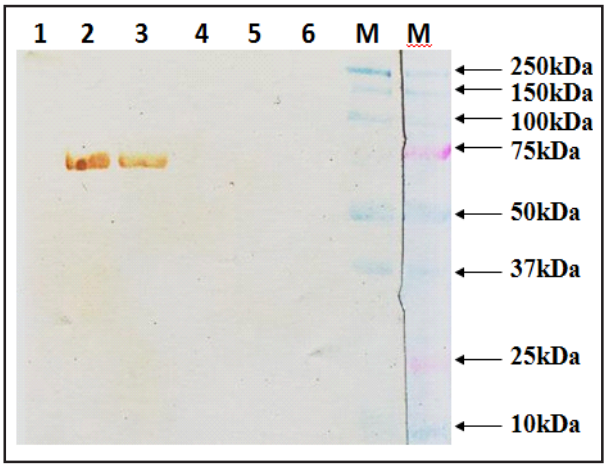

Fig. 6. Western blot analysis of the purified pET/*Htri*HSP 70 protein expressed products (M : Protein molecular weight marker; 1 : BSA; 2, 3, 4 : Expressed products of purified pET/*Htri*HSP 70 protein for 4, 2 and 0 h by IPTG induction; 5 : Expressed products of pET21-b and 6 : Expressed products of purified pET21-b protein).

HSP 70 gene expression, the HSP 70 genes of the larvae after heat shock at 40°C for 30 min were used for prokaryotic expression in this experiment, and the results showed that HSP 70 protein was highly expressed in the *H. tristalis*. The protective effect of HSP 70 on the cell was only functional in a certain scope. On one hand, the high expression of heat shock protein increased the heat tolerance of the insects, free from the direct damage of the temperature stress on the insects; but on the other hand, the induced expression of heat shock protein might affect the normal growth and development of insects, resulting in negative effects on many physiological processes such as reproduction. Insects must be able to maintain a certain balance between the both during the process of adaptation to the external environment, and this balance also reflects the adaptive evolution of the insects on the environment (Cui *et al.*, 2010).

In conclusion, the synthesized heat shock protein of the organisms induced in a variety of stress conditions is an important research topic in biological resilience physiology today (Simoncelli *et al.*, 2010). Although the expression mechanism of HSP 70 gene needs further study, the full-length cDNA sequences of the *H. tristalis* HSP 70 gene were successfully cloned in the above study. The analysis on prokaryotic expression of the transcription and protein levels of the gene at the mRNA level helps to further understand the molecular mechanism of *H. tristalis* larvae adapting to heat stress, but also provides a reference for studying the adaptability of insects on the stress environment.

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