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Full Length Article

Gene Cloning and Gene Expression of Hsp90 from *Meloidogyne incognita* under the Temperature and Heavy Metal Stress

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Abstract

The full length cDNA (2353 bp) of Hsp90 from *Meloidogyne incognita* was isolated through the rapid amplification of cDNA ends (RACE) method. The homology analysis revealed *M. incognita* Hsp90 amino acid sequence shared high similarity with Hsp90s of other eukaryotes. Based on QRT-PCR analysis, it suggested that the relative expression level of *M. incognita* juvenile (J2) improved subjected to heat shock, cold shock or heavy metal stress—a higher Mi-Hsp90 expression level compared with its CK (25°C) subjected to stress at 39°C and still higher than that subjected to cold shock or heavy metal stress—32.47 times as much as its CK's level at 6 h after heat shock and 10.06 times as much as its CK's level at a peak, 1 h after cold shock (4°C), while it was 4.01 times as much as its CK's level at a peak, 24 h after heavy metal stress. The relative Mi-Hsp90 expression level subjected to heavy metal stress was lowest, but it was higher compared with CK whatever stressor there might be. That indicates J2 Mi-Hsp90 different expressions equally when exposed to heat shock, cold shock or heavy metal stress, and will serve communications between plant parasitic nematodes and their environment.

Key words: *Meloidogyne incognita*; Plant parasitic nematode; Hsp90; Gene expression; Temperature and copper stress

Introduction

Heat shock protein (HSPs) is a ubiquitous group of highly conserved molecular proteins that have been characterized in a wide range of organisms including all prokaryotes and eukaryotes organisms from *Escherichia coli*, *Drosophila melanogaster*, *Caenorhabditis elegans* to humans. It is a kind of stress protein specifically generative, subjected to high temperature or other stressors. It is even more apparent in the studies over 40 years that it plays important roles in organic evolution with its far-reaching influence on species evolution (Conner *et al*., 1990; Gupta, 1995; Konstantopoulou and Scouras, 1998; Feder and Hofmann, 1999; Birnby *et al*., 2000; Krishna and Gloor, 2001; Park *et al*., 2005). According to HSP molecular weight such families are classified as HSP macromolecule (110～100KD), HSP90 (83～90KD), HSP70 (68～70KD), HSP60, HSP40 and HSP of low molecule mass (＜30KD) (Lindquist and Craig, 1988; Sørensen *et al*., 2003).

Hsp90 as a protein with the richest expression in eukaryotic cells will induce expressions subjected to manifold stresses. Most of Hsp90 exists in cytoplasm with its minority in nucleus (Schlesinger, 1990). Hsp90, the molecular chaperone, is capable of binding nuclear receptor and steroid hormone receptor, as well as binding several protein kinases molecular (including tyrosine protein kinase and serine/threonine kinase in the signal transduction pathway), regulating their bioactivity and participating in a range of life processes such as mitosis, immunity or signal transduction (Pearl and Prodromou, 2000; Picard, 2002; Pratt and Toft, 2003; Péroval *et al*., 2006; Tsutsumi and Neckers, 2007). Moreover Hsp90 plays important roles in its induced overexpression under such stresses as heat, cold shock or heavy metals or any conditions of sudden changes. Even under non-stress conditions, Hsp90 is still indispensable to eukaryotes when it plays critical parts for cells in their survival in response to physiological and pathological or stress conditions (Borkovich *et al*., 1989; Morimoto, 1998; Krishna and Gloor, 2001; Fangue *et al*., 2006; Gao *et al*., 2008; Kim *et al*., 2009).

Meloidogyne incognita is mostly hazardous to the plant roots of *Solanaceae*, *Cucurbitaceae* and *Cruciferae* where nodular root-knots appear, destructive to root tissue differentiation and physical activity, inhibitive for the normal growth above ground and then the plant yield and quality will be seriously impacted (Liu, 2000; Liu *et al*., 2010, 2011), which will result in yield reduction of 15~30% perennially or more than 80% seriously or even without any harvest for the affected field host (Barker *et al*., 1976). All the organisms including *M. incognita,* whose major

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distribution in sub-tropical area and temperate region, will have to cope with environmental stress and physical stress in the nature (Mcsorley, 2003), of which temperature is the most important element, influential to the distribution of most nematode species worldwide and also another key environmental element for plants in response to *M. incognita* including nematode survival, distribution, embryogenesis and hatching, immigration and invasion, growth and symptom on the plants (Liu, 2000). Therefore a series of protection mechanisms, one of which is to induce relative protein synthesis (Hsp90 for instance), will be developed in the organism to cope with those stresses for its survival (Devaney, 2006).

Study about Mi-Hsp90 is mainly focused on parasitic and free-living nematodes (Birnby *et al*., 2000; Devaney *et al*., 2005; Gillan *et al*., 2009), nevertheless there is few research on molecular and functions of the plant parasitic nematodes. The only report is limited to Hsp90 cloning and its characteristics associated with *Heterodera glycines* (Skantar and Carta, 2004) and *M. artiellia* (Luca *et al*., 2009). Only partial sequence was cloned on the existing DNA basis (GenBank accession number EU364881, 2008), yet there is no further study about *M. incognita* Hsp90.

It is observed in the early study of the research group that ions in soil and the fertilizer applied by the farmers for the purpose of plant adsorption remarkably influenced nematodes' behavior and then influenced field diseases indirectly. As most sensitive to compound of $CuSO_4 \cdot 5H_2O$, a low concentration will cause *M. incognita* J2 mortality (Duan, 2009). Hsp90 was once proposed to take part in cold and heat shock, but the reaction mechanism of Mi-Hsp90 subjected to the temperature or heavy metal stress was not under favorable understanding. Thus full-length cDNA of Hsp90 was isolated individually from *M. incognita* juvenile (J2), and Hsp90 gene expression reaction to heat, cold or heavy metal stressors was detected in the analysis of the ecologically adaptive mechanism of *M. incognita* under stressors.

Materials and Methods

Experimental Details and Treatments

Experimental material: The nematodes were collected from the solar greenhouse of Qiansai Village, Hunhezhan Town, Dongling District, Shenyang. Single oocysts sterilized by 0.5% of NaClO was inoculated into the susceptible tomatoes of L402 variety (The inoculums soil was suffered from dry heat sterilization at 180°C and L402 variety was the gift from Liaoning Academy of Agricultural Sciences) for the purpose of amplification. The culturing temperature was 25°C and 30 d after inoculation, multiple oocysts appeared at the tomato root tissues to be taken out and gently flushed accordingly, so that females and J2 were collected and identified rigorously as *M. incognita*. Upon amplification for the second generation was completed, fresh oocysts were picked from the susceptible tomato root tissues, and sterilized by 0.5% of NaClO for 3 min, flushed by sterile water three times. Then they were placed into the Petri dishes for cultivation in thermostat at 25°C with water renewed once every 24 h to ensure the consistent freshness and vitality of J2 in each selection (Liu, 1995).

Extraction of Total RNA and Synthesis of First Strand of cDNA

When the J2 samples collected were ready, RNA simple Total RNA Kit (Tiangen) was employed in total RNA extraction, for which all the operations conformed with the Kit instructions. Synthesis of first strand of cDNA was conducted according to the RACE Kit (Invitrogen) instructions. Superscript III Invitrogen was employed for first strand of cDNA in RACE experiment.

Primer Design and PCR Reaction

In light of the full-length sequence of relatives (*M. artiellia* GeneBank: FM897369.1). Oligo6.0 and Primer primer5.0 software were applied in specific primer design. After cDNA fragments of *M. incognita* Hsp90 were obtained, RACE (Invitrogen) was adopted in the amplification for 5' and 3' end sequences (PCR reaction primers in the study seen in the following table).

Primer nema	Sequences (5'-3')	Note
Specific primer	MI90-A: 5'-GATGAAGAGCTGAACAAGAC-3 ' MI90-B: 5'-TTAGTCAACCTCCTCCATAC-3 '	own design
3' RACE specific primer	GSP w: 5'- AAGAAGCGAGGTTTTGAAGTTAT -3 ' GSP n: 5'- GGTTTAGAGTTGCCAGAAAGTGA -3 '	own design
3' RACE adapter primer	AP: 5'–GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT–3' AUAP : 5'–GGCCACGCGTCGACTAGTAC–3' UAP: 5'–CUACUACUACUAGGCCACGCGTCGACTAGTAC–3'	3' RACE Kit
Specific primer	Hsp F2 : 5'-ATGAT(A/C/T)GG(T/AC)CA(A/G)TTCGGTGT-3' Hsp R2 : 5'- GTCCTTATCTTCAGCGATTTCAT -3'	own design
5' RACE specific primer	GSP2: 5'-CTTCTTCTCATCCTCAGCCTCAT-3' GSP3: 5'-GCTTCTTAACCACTTCACGAATA-3' GSP4: 5'- CATCTCAGGATCAACGCAGTTAC-3' GSP5: 5'- ACCAAGAAGGCAGAGTAGAAACC-3'	own design
5' RACE adapter primer	Abridged Anchor Primer: 5'–GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG–3' AUAP: 5'–GGC CAC GCG TCG ACT AGT AC-3' UAP: 5'–CUACUACUACUAGGCCACGCGTCGACTAGTAC-3'	5' RACE Kit
Real-time PCR primer	Hsp90-F: TCTCGTGAAATGCTCCAA Hsp90-R: TACGGTTGACAGAATCTTCG	own design
β-actin Reference gene specific primer	18srRNA-F: GATACCGCCCTAGTTCTG 18srRNA-R: CCTTCCGTCAATTCCTTT	own design

The fragment amplification system is as follows: PCR reaction was performed in a 50 μL reaction volume containing 5 μL of 10×PCR Buffer, 1 μL of dNTPs (10 mM), 1 μL of each primer (10 μmol L-1, MI90-A /MI90-B), 37.5 μL of PCR-grade water, 0.5 μL of *Taq* polymerase (Takara), 4 μL of cDNA template. The PCR temperature profile was 94°C for 3 min, 94°C for 30 s, 50°C for 50 s, 72°C for 1 min 30 s. Go to step 2 for 29 cycles, a final

extension step at 72°C for 10 min, 4°C forever, End. Nest PCR was adopted in the amplification for 5' and 3' end with the amplification conditions in reference to RACE Kit instructions. 3' end amplification system: 10×PCR buffer (2.5 μL), Mg^{2+} (25mM) 1.5 μL, dNTP Mix (10 mM) 0.5 μL, GSP w/GSP n (10 μM) 1.0 μL, cDNA (PCR products of the last round) 2.0 μL, AUAP/UAP (10 μM) 1.0 μL, *Taq* DNA polymerase (0.5 μL), DEPC H_2O (16.0 μL), 25 μL system prepared. 94°C pre-degeneration for 3 min, 94°C degeneration for 30 s, 55°C annealing for 30 s, 72°C extension for 2 min. Go to step 2 for 30 cycles, 72°C extension for 10 min, 4°C forever, End. The experiment was divided into two parts due to impact from Hsp secondary structure at 5' end. The first part: a bit of sequences were obtained from amplification based on the primer design across the secondary structure region; the second part: 4 primers were designed in the middle of the sequences obtain from the first part and they were applied in the final amplification at 5' end. Primer design is based on the homology alignment of nematode Hsp90 gene deposited in NCBI (*M. artiellia* GeneBank: FM897369.1, *H. glycines* FJ985783 and *Steinernema feltiae* FJ584283). Reaction System across the Secondary Structure as follow: 10×Taq reaction Buffer (2.5 μL), cDNA 1 μL, Hsp F2 (10 μM) 1 μL, Hsp R2 (10 μM) 1 μL, dNTP 0.5 μL, *Taq* DNA polymerase 0.5 μL, Mg^{2+} (10 mM each) 1.5 μL, ddH_2O 17 μL, 25 μL system prepared. 94°C pre-degeneration for 5 min, 94°C degeneration for 30 s, 55°C annealing for 30 s, 72°C extension for 1.5 min, Go to step 2 for 35 cycles, 72°C extension for 5 min, 4°C forever, End. 5' end amplification system: 10×PCR buffer 2.5 μL, Mg^{2+} (25mM) 1.5 μL, dNTP Mix (10 mM) 0.5 μL, GSP2 (10 μM)/GSP3/GSP4/GSP5 1.0 μL, dC-tailed cDNA (PCR products of the last round) 2.5 μL, Abridged Anchor Primer/AUAP/UAP/AUAP (10 μM) 1.0 μL, *Taq* DNA polymerase 0.5 μL, DEPC H_2O 15.5 μL, 25 μL system prepared.

PCR Product Cloning and Sequencing

The PCR products were separated on 1.0% agarose gel and purified by the PCR fragment purification kit (Axygen). The purified PCR product was ligated into the pGEM-T easy vector (Promege) and transformed into competent *Escherichia coli* cells. Both were considered all right that the direct determination with bacteria liquid or recombinant plasmid extracted for sequencing.

Bioinformatics Analysis of Target Gene

BLAST software and DNA MAN were employed in sequence homology alignment, similarity search, sequence assembly and protein sequence analysis; Clustal W program was employed in multiple sequence alignment; Clustal x and MEGA4.1 software were applied for the construction of a phylogenetic tree with neighbor-joining method.

SYBR Green Quantitative Real-time PCR

Pure lines of fresh J2 were placed into centrifuge tubes of 1.5 mL and then they were treated by $CuSO_4 \cdot 5H_2O$ of 0.0003 $mol \cdot L^{-1}$ prepared with deionized water at 4°C and 39°C individually for 1 h, 6 h and 24 h with centrifugation of 12000 rpm for 20 min. Then the supernatant was discarded and sediment under the same treatment was transferred equally into the clean centrifuge tubes weighed beforehand for a second centrifugation and transfer for further centrifugation once again until the individual nematode weight upon treatment was 20 mg. After that it was subjected to quick freezing with liquid nitrogen and restored in ultra-low freezers at -80°C. Treatment with sterile water was conducted at a room temperature of 25°C as controls, three times for each sample, treatment with compound at a room temperature, treatment at 4°C in the temperature regulating freezer and treatment at 39°C in the thermostatic water bath. RNA simple Total RNA Kit was applied in total RNA extraction and the first strand of cDNA synthesis was based on TIAN Script RT Kit instructions. Exicycler™ 96 fluorescence quantitative device manufactured by Korean BIONEER Company was adopted in the experiment hereby for fluorescence quantitative analysis. The reaction system was as the following: cDNA module 2 μL, 1 μL (10 μM) for Hsp90-F/Hsp90-R respectively, SYBR GREEN mastermix (Tiangen) 9 μL, 20 μL with ddH_2O complement. The reaction condition: predegeneration at 95°C for 10 min, 95°C for 10 s, 60°C for 40 s with Scan added for fluorescence collection once, go to step 2 for 40 cycles. The dissolution curve was defined to rise from 55°C to 95°C in temperatures with the fluctuation of 1°C in the duration of 1s. The product size was detected with agarose gel electrophoresis, of which the internal primer amplification fragment was 153 bp in length, quantitative primer amplification fragment of 175 bp in length. The relative gene expression was subjected to quantitative calculation with the $2^{-\Delta\Delta Ct}$ approach for the relative expression of each treated sample, three times in all.

Statistical Analysis

Discrepancy in data ($P < 0.05$) obtained from quantitative expression was analyzed with the help of analysis software ANOVA of SPSS 13.0 and Duncan multi-comparison test.

Results

Clone the Full-length *M. incognita* Hsp90 cDNA

The primers of MI90-A/MI90-B were used for partial sequence amplification. There was only one light band with the length of 1346bp for the product electrophoresis (Fig. 1A). The sequencing results subjected to BLAST alignment analysis revealed that it shared high similarity with the Hsp90 sequences of other known organisms, and then 3' end sequence of 712 bp was acquired with the help of RACE-

PCR (Fig. 1B). Due to the secondary structure impact from 5' end, firstly primer amplification was designed across such a structure to acquire a sequence of 549 bp (Fig. 1C). Upon three cycles of amplification of 5' RACE followed by the validation and sequencing with bacteria liquid PCR, then the final 5' end sequence of 422 bp was obtained. The discrepancy between GSP4 and GSP5 was no more than 100 bp in primer design, and the electrophoresis results were consistent with the prediction (Fig. 1D), a proof of monoclone-centered positive clone for the gene. Eventually the acquired sequences were subjected to assembly with the help of DNAMAN software to obtain the full-length cDNA of 2353 bp from *M. incognita* Hsp90 (Fig. 2) (GenBank accession number: GU441459). It was named as Mi-Hsp90, whose reading frame was 2127 bp encoding 708 amino acids with the predicated molecular weight of 81.7 KD and isoelectric point of 4.75, 5' untranslated region (UTR) of 78 bp, and 3' untranslated region (UTR) of 128 bp [not including poly (A)].

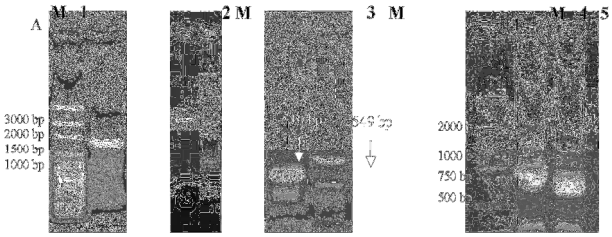

Fig. 1: PCR amplification result of Mi-Hsp90 gene in *Meloidogyne incognita*

Note: M: Marker; 1~5: Amplification products; A: Amplification products using specific primer; B: Amplification products using 3' RACE specific primer; C: Amplification PCR products across the secondary structure region; D: Amplification products using 5' RACE specific primer, electrophoresis channel 4: PCR amplification from bacteria Liquid of GSP4 and UAP primer, electrophoresis channel 5: PCR Amplification from bacteria liquid of GSP5 and AUAP primer

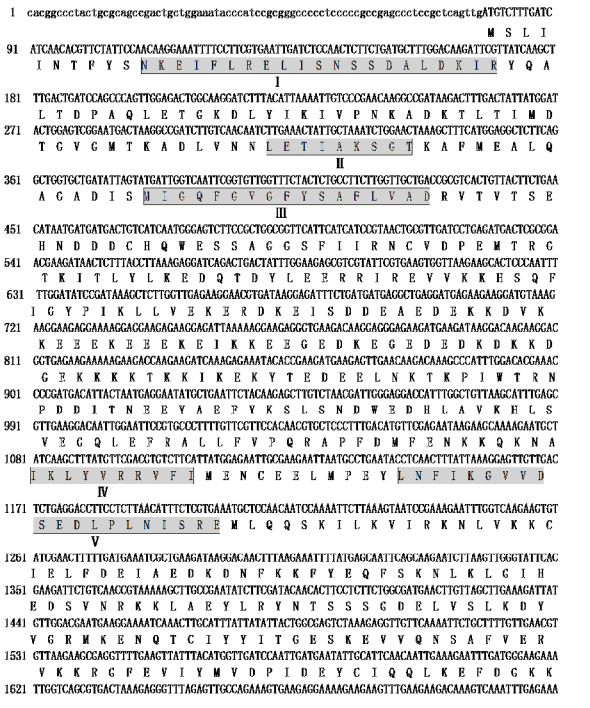

Fig. 2: Nucleotide and deduced amino acid sequence of *M. incognita* Mi-Hsp90 cDNA

Note: Lowercase represents 3' and 5' untranslation region; Capital represents translation region, above Nucleotide sequence, below amino acid sequence; Asterisks mark termination codon; Signature sequences are indicated by **Ⅰ-Ⅴ** Gray fonts; The consensus leucine zipper sequence is underline, which has been shown to bind nuclear receptors, is indicated with a solid bar; The MEEVD motif are double underline

Sequencing Analysis

The deduced amino acid sequence of Mi-Hsp90 included 5 Hsp90 family signatures (Fig. 2), so it was confirmed as one of Hsp90 family members. When the deduced amino acid sequences of Mi-Hsp90 were subjected to alignment against the Hs90s of 8 plant-parasitic nematodes of full-length sequence deposited in GeneBank and Hsp90s of other known representative organisms, the results revealed that the homology between Mi-Hsp90 and *M. artiellia*, *Heterodera glycines*, *C. elegans*, *Brugia pahangi*, *Dictyostelium discoideum*, *D. melanogaster*, *Mus musculus* and *Arabidopsis thaliana* was 89.12%, 85.85%, 77.38%, 81.78%, 60.80%, 70.07%, 72.73% and 63.61%, respectively (Fig. 3). Such a sequence also shared a higher homology (though not indicated statistically) with the Hsp90 of other vertebrates (such as chickens, pigs, chimpanzees and human beings). 5' UTR amino acid sequence of Mi-Hsp90 (or dozens of bp prior to promoters) was different from those of the other two plant-parasitic nematodes newly deposited (Fig. 4)—*M. artiellia* (FM897369) and *H. glycines* (AF461150). Upon repeated tests and sequencing, what was acquired confirmed such a sequence in spite of the mutation at 5' end, different from other species. Moreover the presence of a bit of characteristic motifs (MEEVD) at C-terminal, consensus sequence of leucine zipper（LVVLL） and ATP binding domain at N-terminal were typical of cytoplasm Hsp90 and indicative of the existence of such a protein in cytoplasm.

Phylogenetic Analysis of Amino Acid Phylogeny

A phylogentic tree of Hsp90 was constructed (Fig. 5) by neighbor-joining method with the help of MEGA4.1 software when Mi-Hsp90 encoding amino acid sequences and full-length Hsp of 21 different species selected from GenBank were involved. As indicated in Fig. 5, those belonging to Nematode formed a large branch among the 21 selected species, and *M. incognita* Hsp90, together with *M. artiellia* and *H. glycines* from Nematode Door/Nematoda /Tylenchida/Heteroderidae, formed another branch, where it enjoyed the closest genetic relationship in terms of Hsp90 with *M. artiellia* from the same *M. incognita*, a

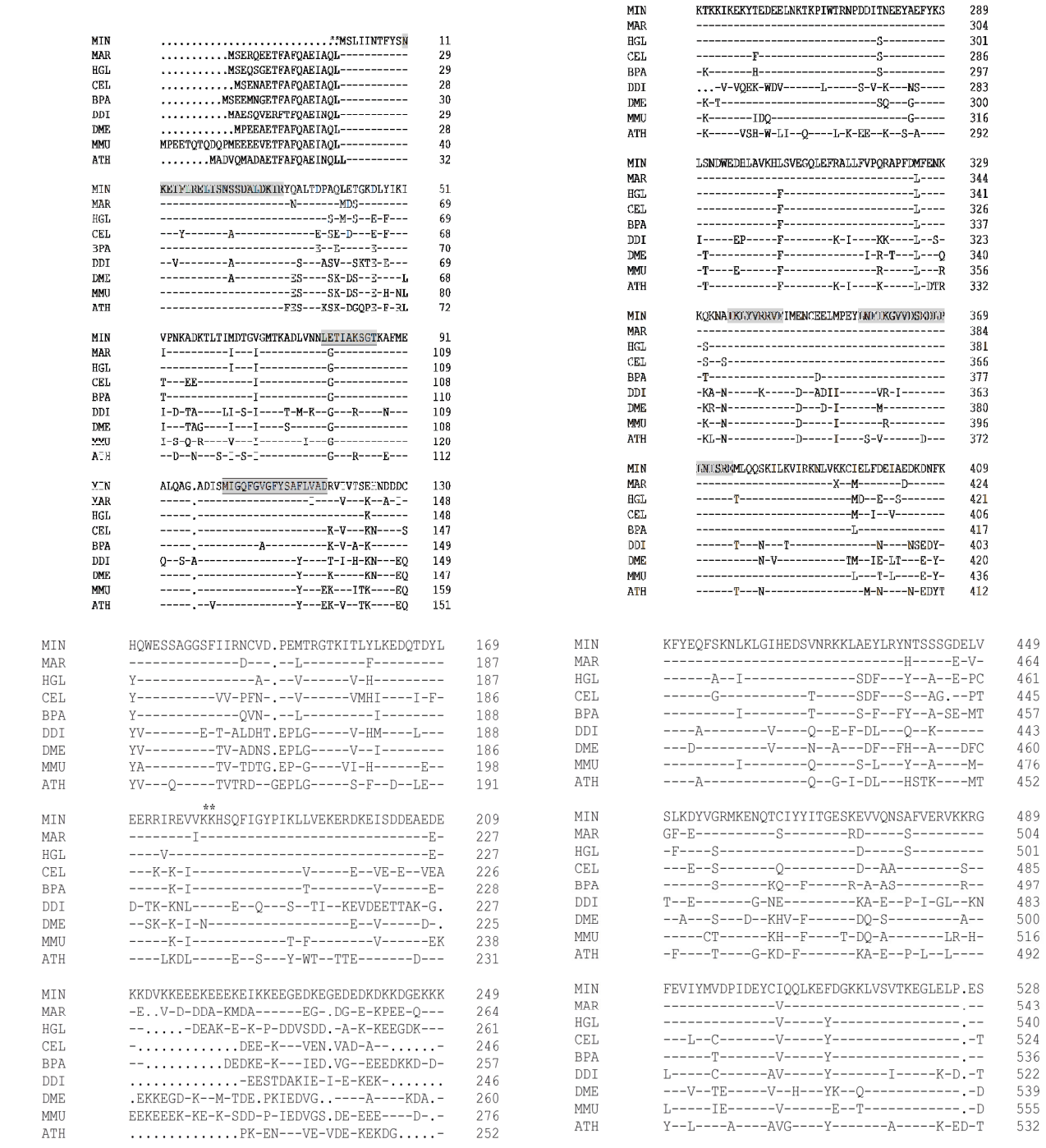

Fig. 3: Multiple alignments of predicted *M. incognita* Mi-Hsp90 translation compared with known Hsp90s

Note**:** MIN: *M. incognita* Hsp90, MAR: *M. artiellia* (CAU15484), HGL: *Heterodera glycines* (ACR57215), CEL: *Caenorhabditis elegans* (CAA99793), BPA: *Brugia pahangi* (O61998), DDI: *Dictyostelium discoideum* (AAA69917), DME: *Drosophila melanogaster* (P02828), MMU: *Mus musculus* (NP_034610), ATH: *Arabidopsis thaliana* (AAA32822), Signature sequences are indicated by Gray fonts; Typical motifs were shown in a gray box; **Denotes the start and the end of the N-terminal ATP-binding domain

Fig. 3: Continued

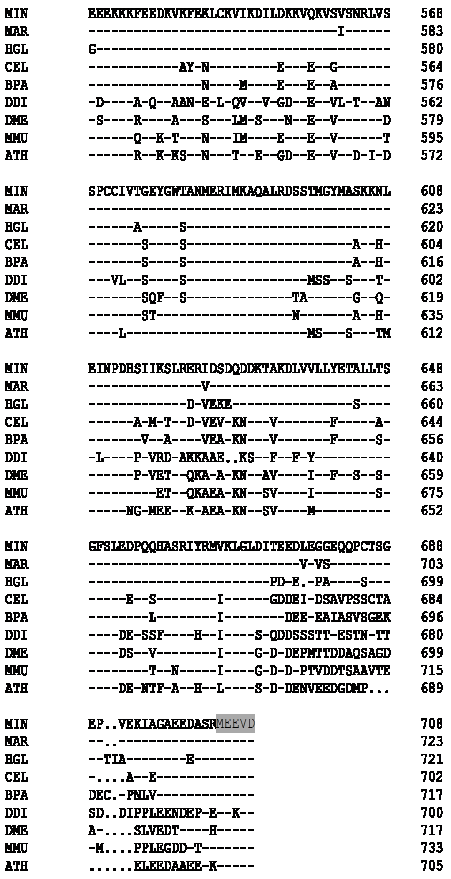

Fig. 3: Multiple alignments of predicted *M. incognita* Mi-Hsp90 translation compared with known Hsp90s

Note：MIN: *M.incognita* Hsp90, MAR: *M.artiellia* (CAU15484), HGL: *Heterodera glycines* (ACR57215), CEL: *Caenorhabditis elegans* (CAA99793), BPA: *Brugia pahangi* (O61998), DDI: *Dictyostelium discoideum* (AAA69917), DME: *Drosophila melanogaster* (P02828), MMU: *Mus musculus* (NP_034610), ATH: *Arabidopsis thaliana* (AAA32822), Signature sequences are indicated by Gray fonts; Typical motifs were shown in a gray box; **Denotes the start and the end of the N-terminal ATP-binding domain

fact in good agreement with its taxonomic status. Such a phylogenetic tree is basically representative of the traditional taxonomic status for nematodes and explanatory for Hsp90 as an appropriate gene in Nematoda phylogeny analysis.

The Relative Mi-Hsp90 Gene Expression of *M. incognita* Subjected to Stresses

The relative expression level of Mi-Hsp90 of *M. incognita* subjected to stresses such as heat shock, cold shock or heavy metal stress was under Real-time PCR analysis. The results displayed a higher Mi-Hsp90 expression level compared with its CK subjected to stress at 39°C, and still higher compared with that subjected to cold shock or heavy metal stress—32.47 times as much as its CK's level, 6 h after heat shock and 10.06 times as much as its CK's level at a peak, 1 h after cold shock (4°C), while it was 4.01 times as much as its CK's level at a peak, 24 h after heavy metal stress. The relative Mi-Hsp90 expression level subjected to heavy metal stress was lowest compared with the other two stresses, but it was higher compared with its CK whatever stressor might be there (Fig. 6 and 7).

Discussion

Hsp90 with its wide distribution in different species is highly conserved. The amino acid residues of the five Hsp90 signature sequences were of few variations as consistent substantially in lower or higher eukaryote (Fig. 3). The homology in a sense of amino acids for Hsp90 from different nematodes was higher than 75%. As the research from Gillan *et al.* (2009) indicated, the amino acid homology was higher than 90% for *C.elegans Daf-21*,

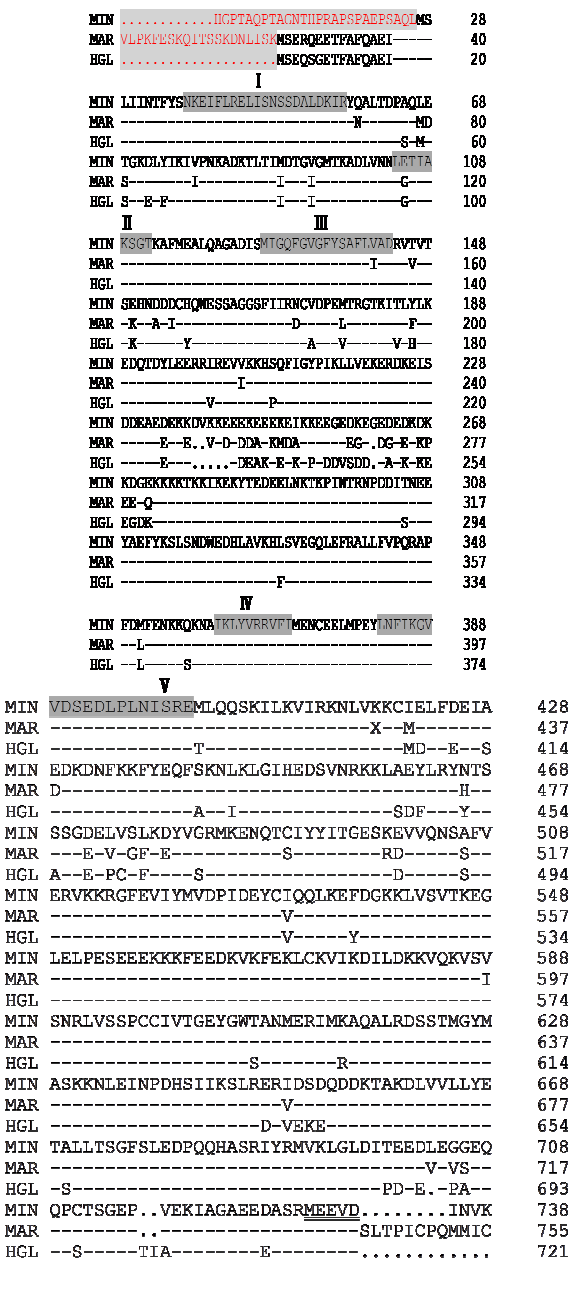

Fig. 4: Hsp90 amino acid alignment of three plant-parasitic nematodes

Note: *M.incognita* (MIN, GU441459), *M.artiellia* (MAR, FM897369) and *Heterodera glycines* (HGL, AF461150) of full-length sequence are translated as amino acid alignment. 5' UTR amino acid discrepancy of the three nematodes are indicated in red letters in light grey block. Signature sequences are indicated by **Ⅰ**-**Ⅴ** shaded in dark grey; The MEEVD motif are double underline

Brugia pahangi Hsp90 and *Haemonchus contortus* Hsp90. When green fluorescent protein (GFP) was employed to observe Hsp90 distribution in nematodes, it was found equally in nematode somatic cells, especially its obvious expression in intestines and nerve rings; moreover swollen intestine, distorted alimentary canal, protruding vulva or underdeveloped Gonad were found after Hsp90 expression was inhibited by RNAi for nematodes. This shows accordingly that Hsp90 was of significance to the life activity of organisms. In the study hereby *M. incognita* as one of important plant-parasitic nematodes was the study object, from which Hsp90 full-length sequence was obtained. The sequence analysis demonstrated that Mi-Hsp90 was typically characterized with eukaryotic cells, for example the five conserved regions concerning NKEIFLRELISNSSDALDKIR, LETIAKSGT, MIGQFGVGFYSAFLVAD, IKLYVRRVF, LNFIKGVVDSEDLPLNISRE and MEEVD motif at C-terminal (Gupta, 1995; Skantar and Carta, 2004; Devaney *et al*., 2005; Gillan *et al*., 2009; Luca *et al*., 2009). EEVD of 4 amino acids at C-terminal were the cytoplasm localization signal for Hsp family and such a peptide identified by TPR domain in HOP (HSP70 and HSP90 organizing protein) would regulate and participate in multi-molecular chaperone complex assembly (Pearl and Prodromou, 2000; Scheufler *et al*., 2000; Gaiser *et al.*, 2009). As predication based on the above sequence analysis confirmed, Mi-Hsp belonged to Hsp90 family in a cytoplasmic sense and homologous to *C.elegans Daf-21* (Birnby *et al.*, 2000). Mi-Hsp90 encoding amino acids shared high similarity with other known Hsp90s, especially with *M. artiellia* belonging to the same *M. incognita*, for the homogeneity between them was 89.12%. Hsp90 topological structure was of Oligo-fit with a symmetric structure on both sides at N-terminal and the interval between N-terminal and C-terminal was characterized with APT-binding and substrate-binding (Huai *et al*., 2005; Pearl *et al*., 2006). The alignment results for Mi-Hsp90 versus other species revealed that ATP domain and substrate-binding domain were rather conserved in all the species. Though amino acid sequence subjected to heat shock was conserved with slight encoding mutation, such characteristics as heat shock protein sequence and copy number were under constant changes with the species evolution. In the findings of the study hereby, full-length 5' UTR sequence of Mi-Hsp90 was different from other deposited 5' UTR of *M. artiellia* and *H. glycines* for instance, namely mutations occurred, a fact potentially associated with the specie itself or its geographical locations, or test conditions (Seen in Fig. 4).

Hsp90 conservation may serve as a definite scientific basis in the biological evolution analysis. A phylogenetic tree of a representative group of nematodes was constructed when Hsp90 was applied in the essay. The research results were identical with those of *H. glycines* Hsp90 (Skantar and Carta, 2004) analysis by SSU（small subunit）approach (Blaxter *et al.*, 1998). Therefore the research hereby will be of a reliable basis for the systematics and evolution study of Nematoda in the future.

The relative expression level of Mi-Hsp90 subjected to cold shock (at 4°C) , heat shock (at 39°C) or heavy metal stress ($CuSO_4 \cdot 5H_2O$) for 1 h, 6 h or 24 h respectively was

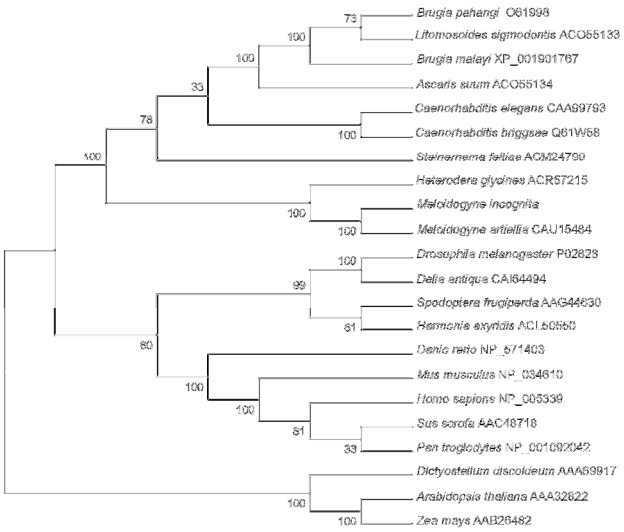

Fig. 5: The phylogenetic tree of relationship of based on Hsp90 by the neighbor-joining method

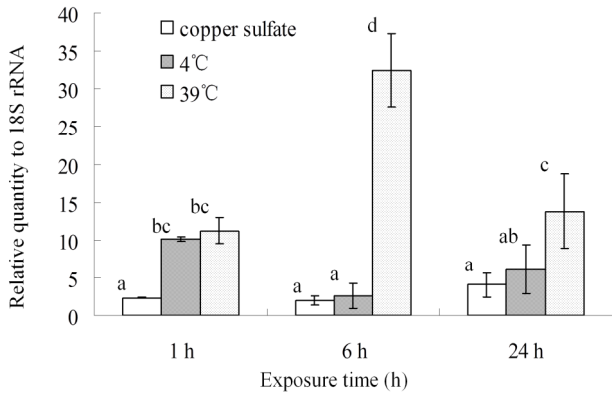

Fig. 6: The relative expression level of Mi-Hsp90 under 4°C heat shock, 39°C cold shock and copper sulfate stress
Note: Values are expressed as mean ±SD (n=3), different letters above each bar indicate statistical difference (P＜0.05)

performed by real-time fluorescent quantitative PCR method. The results displayed that Mi-Hsp90 expression reached to a peak at 1 h after cold shock, 10.06 times as much as its CK's level, a peak at 6h after heat shock, 32.47 times as much as its CK's level, while a peak at 24 h after heavy metal stress, 4.01 times as much as its CK's level, namely different peaks for different stresses. As it was quite illustrative, when organism subjected to external stimulus, oxidation-antioxidation imbalance induced response from organism—a high expression of Hsp90 induced to cope with injuries to organism from stimulus, and it also revealed that Hsp90 played an important role for *M. incognita* J2 in its adaption to temperature and heavy metal stress. In the study from Luca *et al.* (2009), a high expression of Mt-Hsp90 was observed for *M. artiellia* J2 and the egg mass when coping with coldness and high temperature. Additionally in

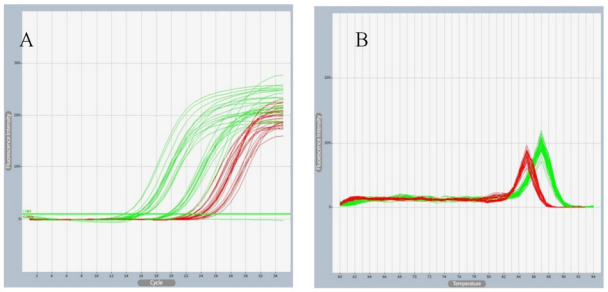

Fig. 7: The fluorescence curves (A) and melting curves (B) of SYBR Green Quantitative Real-time PCR detection on Mi-Hsp90 under 4°C heat shock, 39°C cold shock and copper sulfate stress

the study from Golombieski (2008), Hsp83 expression of *D. melanogaster* as Se-tolerance was observed, these facts much similar with our test results.

The transcript of Hsp90 mRNA subjected to heat shock depended on the heat shock duration and temperature—namely Hsp90 transcript accelerated in a specific shock duration or at a specific shock temperature, while Hsp90 expression was identical with its mRNA level variations, also dependent on the heat shock duration and temperature (Miller *et al*., 1992). Such was our experiment result that Mi-Hsp90 expression was different subjected to different stresses at different periods. In the study from Luca (2009), Mt-Hsp90 expression level was different subjected to the same treatments at different stages though, or J2 Mt-Hsp90 expression was higher than that of its egg mass subjected to heat shock (at 30°C), while egg mass Mt-Hsp90 expression was higher than that of its J2 subjected to cold shock (at 5°C), and still higher than those of J2 and CK at 24 h after treatment. It was illustrative that Mt-Hsp90 played an important role under cold stress for *M. artiellia* egg mass to improve its survivability. In the study from Rinehart (2007) though the expression of Hsp23 and Hsp70 from Drosophila were inhabited by RNAi, Drosophila still experienced its diapause, while such an expression also played a critical part in pupa survivability at lower temperatures and it was believed that Hsp was of significance for the insects in their diapause over winter as cold-resistance, much universal in dormancy regulation and control. The anti-stress mechanism of *M. incognita* egg mass was not detected in our experiements, however it was still indicative of the results that there was a higher mRNA expression of Mi-Hsp90 in J2 subjected to heat shock than cold shock similarly. Whether it was associated with the capability of infection and damage for *M. incognita* J2 at high temperatures will be under further study.

The improved Hsp expression made organisms have to pay for their tolerance to stresses somehow (Hoffmann, 1995; Butov *et al*., 2001), for example poor reproduction, more energy consumption or shorter life. As our previous

study discovered, oocyst hatchability of *M. incognita* declined at 4°C and 39°C with the relative hatching inhibition more than 95%. Meanwhile J2 survivability subjected to stress at 39°C, 24 h after treatment declined (with the data to be published separately), while copper sulfate was a sort of sensitive compound for J2 (Duan, 2009). Hsp90 synthesis speed acceleration was associated with stress intensity—with the stress increasing, Hsp90 synthesis increased, but it turnd to a decline when the critical temperature tolerant for the cells appeared. During the early stress, a higher Hsp90 expression level improved cell survival under unfavorable conditions, while a lower Hsp90 subjected to stress was mostly due to overstress or longer duration, resulting in server damage to nucleus organism. Whether hatchability and survivability decline for *M. incognita* subjected to stress were associated with Hsp90 expression will be under further study.

Subjected to different stressors (such as high temperature, low temperature, drought, heavy metals, OFR, ultraviolet radiation, hypoxia, salt stress, bacterial infection or pesticides), one of the most remarkable physiological changes in the organisms is the changes in Hsp gene expression. Dissimilar with other Hsps, Hsp90 will account for 1%~2% of the total cytoplasmic protein in unstressed eukaryotic cells. The studies suggest that Hsp90 functions physiologically in a wide range (Fliss *et al*., 2000; Pratt and Toft, 2003). The major Hsp90 function is to serve as molecular chaperone, interacting with multiple proteins (Mayer and Bukau, 1999; Pearl and Prodromou, 2000; Pearl *et al*., 2006). It is not only contributory to maintain protein folding and its proper folding pattern in case of adversity, but supportive to decompose misfoling or denatured proteins in the organisms as well to prevent cell loss (Yonehara *et al*, 1996). Hsp90 is a key component in stress biology. The successful cloning of Mi-Hsp90 gene of *M. incognita* in addition to the expression discrepancy determination subjected to different stressors at different period after treatment will not only provide a basis for the further study of such a gene in terms of its expression variations subjected to different treatments, durations and stages, but also pave a way for the intensive study of the adaptive mechanism of *M. incognita* to high temperature and other stressors. It also demonstrates that Hsp90 as apotential bio-indicator will be applied with Hsp70 at the same time to reflect the environmental influence on organisms more accurately. Since Hsp is of a gene family, the adaptability to environmental stress for *M. incognita* will not be confirmed by the expression, variation and induction from only one Hsp gene expression admittedly. Therefore, the relativity between other Hsps, especially the relativity between Hsp90 temporal and spatial gene expression together with dynamic variations and *M. incognita* anti-stress still needs further intensive research. Such will help us to understand how *M. incognita* is adaptive to its environment from a point view of molecular biology for a better ecological control of *M. incognita*.

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