VERSITA

Biologia **64**/1: 102—106, 2009
Section Botany
DOI: 10.2478/s11756-009-0008-8

Molecular cloning and characterization of novel heat shock protein 90 gene from a wild *Vitis pseduoreticulata* native to China

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Abstract: Heat shock protein 90 (Hsp90), known as molecular chaperone, is involved in protein folding and assembly in the cell. In the present study, a full-length cDNA named *Vitis pseduoreticulta* heat shock protein 90 (*VpHsp90)* (GenBank accession Number:EU239815), encoding a heat shock protein 90, was obtained by degenerated primers and 3'-and 5'-RACE from *Vitis pseudoreticulata* according to our previously obtained EST sequence (GenBnak accession number:DV182112), putatively known as *Hsp90*. Comparison of *VpHsp90* sequence has revealed that an open reading frame (ORF) consists of 2,100 bp nucleotides and the translated proteins of 699 amino acid residues. The molecular mass of *VpHsp90* calculated from the deduced amino acid sequence was 80.2 kDa, Isolectric Point was 4.893, which is in close proximity of *Hsp90*. The maximum similarity of *VpHsp90* at nucleotides level (85%) and protein level (96%) was found to be with *Nicotiana tabacum.* Phylogenetic tree analysis at both the nucleotides and amino acids levels indicates that *Vitis pseduoreticulata*, *Nicotiana tabacum,* and *Arabidopsis thaliana Hsp90* sequences comprise one clade, which is closely related to *Oryza sativa, Hordeum vulgare* and *Triticum aestivum Hsp90*s. It may be reasonably concluded that *VpHsp90* possesses the ancestral gene of *Hsp90* similar to that of higher plant species.

Key words: *Vitis pseduoreticulta*; heat shock protein 90 (Hsp 90); gene cloning; Chinese wild *Vitis* species; grapevine

Introduction

In higher eukaryotes, Hsp90 associates with a large set of co-chaperones to mediate the conformational regulation of tyrosine kinases and steroid hormone receptors (reviewed in Buchner 1999; Picard 2006; Young et al. 2001). Hsp90 has also been proposed to buffer phenotypic variation of these signalling molecules, allowing cells to maintain a wild-type phenotype in the face of genetic mutations (Sangster et al. 2004). The structures of Hsp90 and several Hsp90 cofactors have recently been elucidated (Pearl & Prodromou 2006). Previous studies suggest that Hsp90 is not a general chaperone for newly synthesised proteins using biochemical and genetic approaches. Additionally, Hsp90 levels are highly induced by abiotic stress, it is required neither to confer thermotolerance nor to prevent the aggregation of heat-inactivated proteins (Nathan et al. 1997).

Characterization of Hsp90 gene sequences from a more diverse range of plant species would provide a means for better understanding of the molecular evolution and function of Hsp90 also in *Vitis* species. In this research, using 5'-and 3'-RACE, the full-length *VpHsp90* gene from Chinese wild *Vitis* species was firstly cloned from the *Vitis* genus plant based upon GenBank data. Here we characterise the first full-length sequence of *VpHsp90* from a wild *Vitis pseudoreticulata* native to China.

Material and methods

Plant materials and treatments

The grape materials of Chinese wild *V. pseudoreticulata* clone Baihe-35-1, highly resistant to powdery mildew and *V. adstricta* Hance clone Taishan-2, highly susceptible to powdery mildew, maintained in the grape germplasm resources orchard, Northwest A & F University, Yangling Shaanxi, People's Republic of China, were used for the present study. The powdery mildew inoculation was carried out under natural field conditions by pressing infected leaves with *Uncinula necator* of Taishan-2 against the uninfected leaves of Baihe-35-1, pre-sprayed with sterile water, from 8:00 am to 10:00 am on July 8, 2003. The inoculated leaves were immediately covered with paper bags for infection, and the leaves only sprayed with sterile water were used as control.

General DNA techniques

All enzymes for DNA manipulations were purchased from TaKaRa Biotechnology Co. Ltd (Dalian P.R. China) and applied according to manufacturer's instructions.

Escherichia coli DH5a (Amersham Biosciences, USA) strains were used for cloning plasmid constructions. Plasmid DNA was isolated using the plasmid extraction kit (TIANGEN, Beijing, China), DNA fragments were isolated using DNA agarose gel cleanup kit (TIANGEN, Beijing, China).

Isolation of RNA

At 1 d, 2 d, 3 d, 4 d, 5 d, 6 d and 7 d post inoculation, leaves were collected from *V. pseudoreticulata* clone Baihe-35-1 and snap frozen in liquid nitrogen. Total RNA was

isolated from above leaf samples by a partially modified Zhang's SDS/phenol method (Zhang et al. 2003). The purity and integrity of total RNA was confirmed spectrophotometrically and by denaturing agarose gel electrophoresis, respectively.

5'-and 3'-RACE of specific cDNA fragment
In order to obtain the full-length cDNA sequence of a novel gene, 5'-and 3'-RACE was carried out. The gene specific primer HSPR: 5'-CTTGTCCAGAGCATCGGAGGCATT G-3' for 5'-RACE; HSPS1: 5'-CCTCCTCTGTGTTCGGA AATGGCGG-3' and HSPS2: 5'-ACCAGTTGGAATACCT TGAGGAGCG-3' for 3'-RACE were designed according to the sequence of the specific EST sequence (accession No.: DV182112). Total RNA of Baihe-35–1 leaves inoculated with *Uncinula necator* after 7d was isolated as described above and 5'-and 3'-RACE was performed according to the manufacturer's instructions (BD SMART^{TM} RACE cDNA Amplification Kit). The 5'-and 3'-RACE products were separated by a 1.2% agarose gel electrophoresis and purified with DNA agarose gel clean up kit (TIANGEN, Beijing, China). The 5'-and 3'-RACE fragments were cloned into pGEM-T Easy vector (Promega, USA), and sequenced by Takara Biotechnology Co. Ltd.

Sequence analysis of the full-length cDNA
The full-length cDNA sequence with 2,100 bp was obtained by overlapping sequences of 5'-RACE fragment and 3'-RACE fragment containing polyA tail. Homologous sequence searches were performed by blastn and blastx (http://www.nebi.ntm.nih.gov/blast) (Fig. 4). Analysis of *VpHsp90* was performed using MegAlign program of Lasergene software (DNAstar Inc., USA). The neighbour joining tree of *VpHsp90* at both the nucleotides (Fig. 5) and amino acids (Fig. 6) levels were drawn by MEGA (Version 3.1) program. The reliability of each branch of the tree was evaluated using 1000 bootstrap replication. Identity search for nucleotides and translated amino acids was carried out using National Center for Biotechnology Information (NCBI) BLAST network server (http://www.ncbi.nlm.gov/BLAST); *VpHsp90* protein domain was predicted using SMART software (http://smart.embl-heidelberg.de/) (Fig. 7).

Results

5'-RACE of specific cDNA fragment
5'-RACE was employed to obtain the length of 242 bp cDNA sequence. A total of 6.0 μl aliquots of the amplification products were separated on a 1.2% agarose gel after 5'-RACE (Fig. 1). 5'-RACE product was about 250 bp and then cloned into pGEM-T Easy vector, transformed into *E.coli* strain DH5α. The positive clone, characterised by blue/white screening and *Eco*R I digestion, was sequenced by Takara Biotechnology Co. Ltd. It was actually 242 bp in length.

3'-RACE of specific cDNA fragment
3'-RACE was firstly performed to obtain the length of 807 bp cDNA sequence. A total of 6.0 μl aliquots of the amplification products were separated on a 1.2% agarose gel after 3'-RACE (Fig. 2). 3'-RACE product was about 800 bp and then cloned into pGEM-T Easy vector, transformed into *E.coli* strain DH5α. The positive clone, characterised by blue/white screening and *Eco*R I digestion, was sequenced by

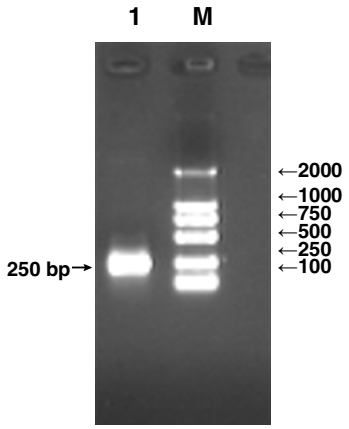

Fig. 1. 1.2% agarose gel electrophoresis: Lane 1: purified 5'-RACE product. M: DNA Makers.

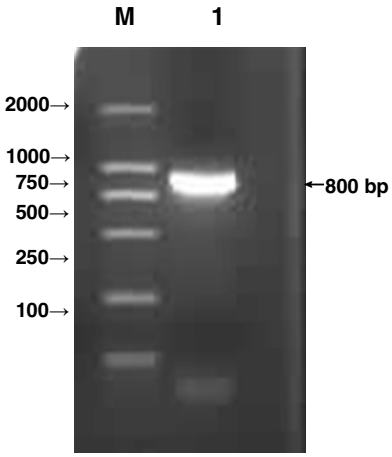

Fig. 2. 1.2% agarose gel electrophoresis: Lane 1: purified first 3'-RACE product. M: DNA Makers.

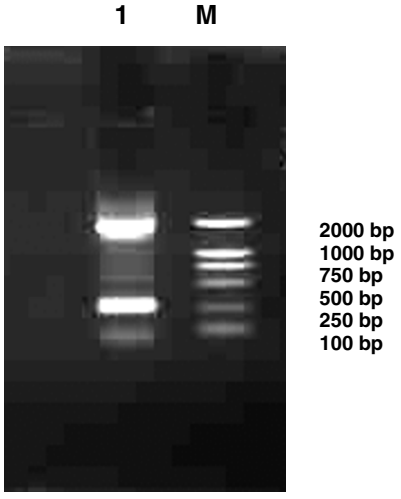

Fig. 3. 1.2% agarose gel electrophoresis: Lane 1: purified second 3'-RACE product. M: DNA Makers.

Takara Biotechnology Co. Ltd. It was actually 807 bp in length. A nonfull-length cDNA with 896 bp was obtained by overlapping sequences of 5'-RACE fragment and first 3'-fragment without 3'-end. Based on this 896 bp cDNA sequence, a second 3'-RACE was carried out. The gene specific primer HSPS2: 5'-ACCAGTTGGAATACCTTGAGGAGCG-3' for 3'-RACE was designed to obtain the full-length of 3'-end cDNA sequence (Fig. 3). 3'-RACE product was about 1,800 bp and then cloned into pGEM-T Easy vector, transformed into *E. coli* strain *DH5α*. The positive clone, characterised by blue/white screening and *Eco*R I digestion, was sequenced by Takara Biotechnology Co. Ltd. It was actually 1,796 bp in length containing polyA tail.

Sequence analysis of the full-length cDNA

One full-length cDNA with 2416 bp was obtained by overlapping sequences of 5'-end 896 bp cDNA fragment and second 3'-RACE 1,796 bp cDNA fragment containing the polyA tail, which is designated as *VpHsp90*. The full-length cDNA sequence has been submitted into GenBank (accession no.EU239815). It contains a 2,100 bp open reading frame that encodes 699 amino acids, and contains 5'-UTR and 3'-UTR and a polyA tail Fig. 4. The molecular mass of Vp Hsp90 calculated from the deduced amino acid sequence was 80.2 KDa; Isolectric Point was 4.893, which is in close proximity of Hsp90. The maximum similarity of *VpHsp90* at both the nucleotides level (85%) and proteins level (96%) was found to be with *Nicotiana tabacum.* Vp Hsp90 protein containing a HATPase_c domain was scanned using SMART software in N-terminus amino acid sites ranging from 27 to 182. Its E-value is 9.40e-10, which is found in several ATP-binding proteins for example: histidine kinase, DNA gyrase B, topoisomerases heat shock protein HSP90 phytochrome-like ATPases and DNA mismatch repair proteins (Fig. 7). The results of the identity analysis and the domain scanning suggest that *VpHsp90* gene is similar to that of known *Hsp90s* in other species. Both the nucleotide and the amino acid sequence comparison showed that *VpHsp90* is closer to *Hsp90* sequence than any other sequence. Phylogenetic tree analysis at nucleotides and amino acids levels indicates that dicotyledons *Vitis pseduoreticulata*, *Nicotiana tabacum* and *Arabidopsis thaliana Hsp90* sequences comprise one clade, which is closely related to monocotyledons *Oryza sativa, Hordeum vulgare* and *Triticum aestivum Hsp90*s; but which is only distantly related to animals (Figs 5, 6). It may be reasonably concluded that *VpHsp90* possesses the ancestral gene of *Hsp90* similar to that of higher plant species.

Discussion

Molecular chaperones are proteins that have evolved to help protein folding inside cells (Frydman 2001). Folding can take place in the cytosol, endoplasmic reticulum, and mitochondria, with each compartment containing a set of highly conserved chaperone proteins

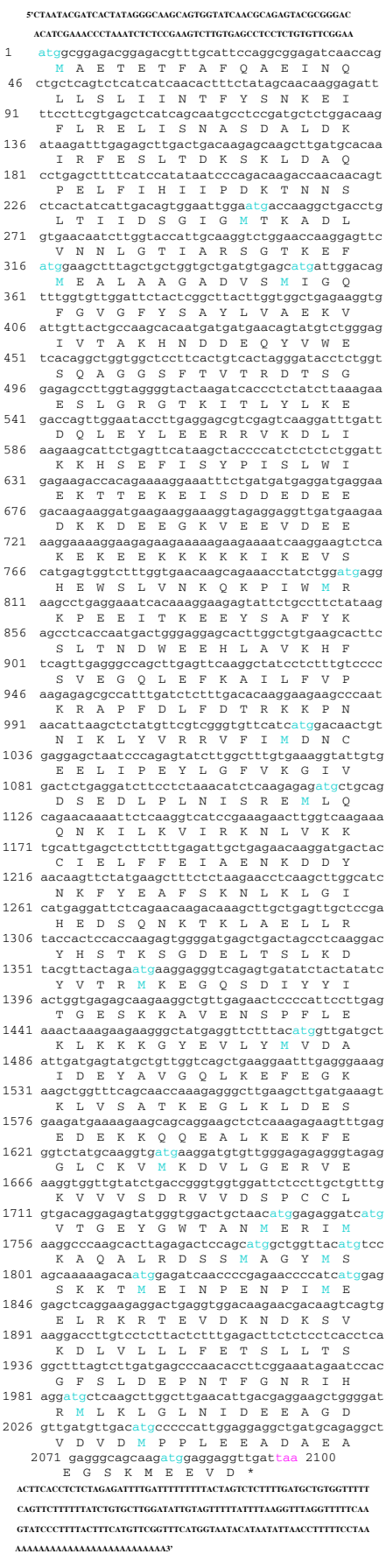

Fig. 4. Nucleotide and amino acid sequence of *VpHsp90* (EU239815). The nucleotide sequence is numbered on the left. 5' UTR and 3' UTR are typed in bold face; * Stop codon

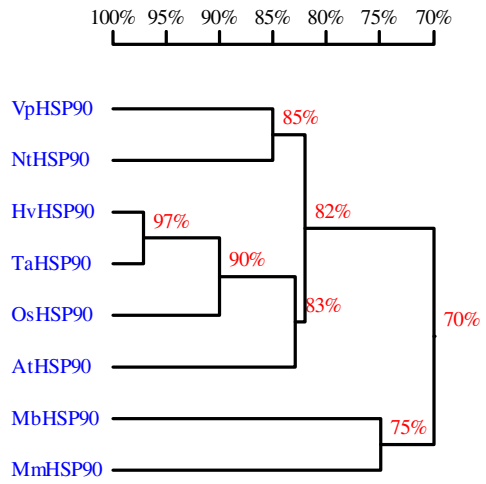

Fig. 5. Phylogenetic tree of the *Vp Hsp90* nucleotide sequence with HSP90s of other species. Notes: *VpHSP90* (); *NtHSP90* (AY519499); *HvHSP90* (AY325266); *TaHSP90* (DQ665783); *OsHSP90* (AB111810); *AtHSP90* (AY081302); *MbHSP90* (AB251894); *MmHSP90* (BC088985).

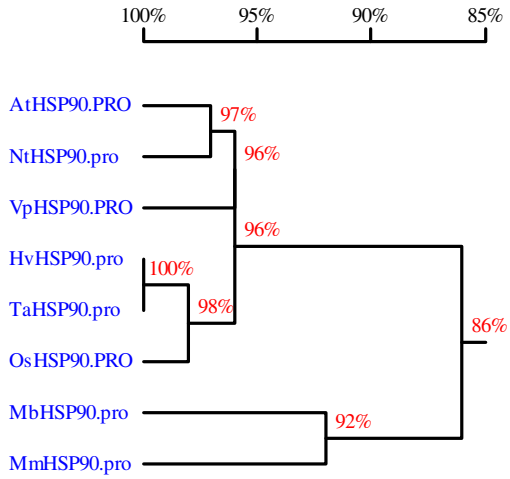

Fig. 6. Phylogenetic tree of *Vp Hsp90* peptide sequence with Hsp90s of other species. Notes: VpHSP90; NtHSP90 (AAS79798); HvHSP90 (AAP87284); TaHSP90 (ABG57075); OsHSP90 (BAD04054); AtHSP90 (AAL91191); MbHSP90 (BAF03554); MmHSP90 (AAH88985).

Fig. 7. *VpHsp90* confidently predicted domains, repeats, motifs and features with SMART software.

of the Hsp70 and Hsp90 families. Chaperones affecting proteins known to promote cell growth and division, including protein kinases and transcription factors (reviewed by Whitesell & Lindquist 2005), are therefore attractive targets for chemotherapeutic agents. Hsp90 associates with a variety of co-chaperones in an ATP-dependent manner to facilitate proper stability and function of client proteins such as the GR (Pratt and Toft 2003). Many studies have demonstrated that Hsp90 activity is regulated by reversible acetylation, a posttranslational modification often associated with histones and chromatin, and an important mechanism by which protein activities are regulated (Kovacs et al 2005). The first inhibitor of Hsp90 described was the natural product geldanamycin (GA), used as a model compound for Hsp90 inhibition. GA and its synthetic derivatives bind to the N-terminal ATP-binding pocket of Hsp90.This results in an inhibition of ATP binding and hydrolysis that are essential for the folding of the client proteins of Hsp90 (Kamal et al 2003). Recent interest in Hsp90 inhibitors as chemotherapeutic agents is based on their ability to promote degradation of client protein kinases through the ubiquitin proteasome pathway (Avrom et al 2006).

When compared to the animal kingdom, the Hsp90/Hsp70-based chaperone machinery of plants is similar but not identical to that of animal cells. Though the predicted amino acid sequences of plant Hsp70s and Hsp90s show identities with those of the human homologues in the range of 70%, the functions of Hsp70 and Hsp90 are highly conserved. (Stancato et al 1996; Dittmar et al 1997). Thus, the two essential chaperones from the plant kingdom have conserved the ability to interact functionally with the co-chaperones of the animal kingdom and to cooperate with them in the protein folding machinery. In addition, there is evidence that TPR protein binding to Hsp90 is involved in the targeted uptake of proteins into glyoxysomes, a class of peroxisomes found primarily in germinating seedlings in plant (William et al 2001; Olsen & Harada 1995). To date, these studies suggest that the Hsp90/Hsp70-based chaperones play a pivotal role in the process of growth and development both in the animal and the plant kingdom.

In this study, based on an EST sequence (accession no.DV182112) previously obtained from cDNA library (Xu et al. 2006), we cloned the full-length cDNA of the novel gene from *Vitis pseudoreticulata* clone Baihe-35-1 with 5'-and 3'-RACE. As described in the sequence analysis above, *VpHsp90* is a novel gene related to heat shock protein 90. Conserved domain, HATPase_c domain (SM00387) (Fig. 7), was found in the deduced amino acid sequence. This single domain family represents the N-terminus (approximately 156 residues) of a number of plants' and animals' Hsp90s.

The deduced molecular mass of 80.2 kDa of cloned *VpHsp90* cDNA is in close proximity to the Hsp90 group from different species. The initial methionine codon is in optimal Kozak consensus sequence (GAAATGG) for translation initiation. Molecular phylogenetic tree based on nucleotide sequences is similar to that of amino acids; it suggests that all the Hsp90 members originated from the same ancestral origin, which has subsequently diverged at different phases of evolution. Dicotyledons *Vitis pseduoreticulata*, *Nicotiana tabacum* and *Arabidopsis thaliana Hsp90* sequences comprise one clade, which is closely related to monocotyledons *Oryza sativa, Hordeum vulgare* and *Triticum aestivum HSP90*s; but which is only distantly

related to animals (Figs 5, 6). It may be reasonably concluded that *VpHsp90* possesses the ancestral gene of *Hsp90* like that of higher plant species. The *VpHsp90* is evolutionarily closely related to the *Nicotiana tabacum* and *Arabidopsis thaliana Hsp90,* suggesting duplication of the same ancestral gene and subsequent diversification in dicotyledons. The divergence of nucleotides and amino acid residues among these three dicotyledons supports this suggestion. The diversity in mature peptide shows evolutionary distance between these species although they come from a common ancestor. This is the first report on the cloning of the full-length *VpHsp90* from the *Vitis* genus plant, based on GenBank data. It could inspire further studies of the multifaceted functions of *VpHsp90* in *Vitis* genus plant, especially in Chinese wild *Vitis* species in the future.

Acknowledgements

This research was supported by National Natural Science Foundation of the People's Republic of China (No. 30571280, No. 30700547) and Provincial Natural Science Foundation of the Shaanxi (No. 2005C$_1$24); the author is also grateful to Dr. Wang Xiangfeng (Department of Molecular, Cellular & Developmental Biology, Yale University) for previously reviewing this manuscript.

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Received November 5, 2007
Accepted August 28, 2008