Genome-wide analysis of the heat shock protein 90 gene family in grapevine (*Vitis vinifera* L.)

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Abstract

Background and Aims: Heat shock protein 90 (Hsp90) proteins constitute an important gene family of molecular chaperons. High-temperature stress, which is often combined with drought stress, may exert major constraints to grapevine growth and development. The aim of this study was to characterise the *Vitis vinifera* Hsp90 gene family.

Methods and Results: Using the complete grapevine genome sequences, four cytoplasmic and three organelle-specific *V. vinifera* Hsp90 (VvHsp90) proteins were identified. Phylogenetic analysis revealed that they share high sequence similarity with their Arabidopsis counterparts, while the cytosolic isoforms are clustered into two distinct groups (VvHsp90.1 and VvHsp90.2). Transcriptional analysis showed that a representative gene from the first group (*VvHsp90.1a*), in contrast to *VvHsp90.2a*, is induced by heat shock in all vegetative tissues/organs tested. Interestingly, it was also expressed in tendrils in the absence of stress. The severity and duration of heat stress influenced in a complex manner the expression profile of *VvHsp90.1a*, while the other *VvHsp90s* tested were rather constitutively regulated. However, the endoplasmic reticulum-specific *VvHsp90.7* was mildly and transiently induced by a relatively prolonged heat stress. Combined drought with heat stress resulted in a delay in *VvHsp90.1a* induction.

Conclusions: Gene structure organisation and expression characteristics of *VvHsp90s* resemble those of their Arabidopsis orthologs, although species-specific differences also exist. Differential regulation of genes suggests functional diversification among isoforms.

Significance of the Study: This is the first report on the characterisation of Hsp90s in grapevine. The present study contributes to a deeper understanding of the complex molecular responses of grapevine to stress.

Abbreviations

Chr chromosome; **ER** endoplasmic reticulum; **GAPDH** glyceraldehyde-3-phosphate dehydrogenase; **MW** molecular weight; **RT-PCR** reverse transcription polymerase chain reaction; **VvHsp90** *Vitis vinifera* heat shock protein 90.

Keywords: *drought, gene expression, heat shock, Hsp90, molecular response, stress protein*

Introduction

Grapevines often encounter environmental stress during their life cycle, which may perturb the physiological status of cells, affect proper development and consequently exert constraints on grape yield and quality. Extreme temperatures are among the most significant factors that limit the distribution of grapevines around the world (Cramer 2010). This particularly concerns cold climates, as grapes are generally tolerant to relatively high temperatures. Thus, winegrowing expands even in very warm regions, such as areas of the Mediterranean basin, where during the growing season, daily temperatures often exceed 40°C. While such high temperatures do not appear to affect significantly the productivity of local varieties, international cultivars of great economic value, especially those originating from cooler climates, may not withstand heat-stress conditions (White et al. 2006, Banilas et al. 2009, Schultz and Stoll 2010). Under the light of upcoming climate changes, like global warming and changes in precipitation patterns, a deeper knowledge of the grapevine physiological response to thermal stress is of particular importance for the worldwide sustainability of viticulture.

Grapevines, like other plants, have developed several strategies to cope with adverse conditions. Those mechanisms include changes in their morphological and/or physiological state to adapt and acclimatise to unfavourable conditions. Upon stress exposure, plants respond by activating the expression of

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specific genes-encoding proteins referred to as 'stress proteins'. Heat shock proteins (Hsps) are such stress proteins that form a superfamily of molecular chaperones having various important functions in diverse organisms, including plants. Molecular chaperones are key proteins contributing to cell homeostasis under both stress and optimal conditions. Hsps were originally identified as intracellular self-defence molecules that are produced in response to heat shock to prevent other proteins from misfolding and aggregation (Lindquist and Craig 1988) or to facilitate their proper refolding under conditions of denaturing stress (Young et al. 2001). The role of Hsps, however, extends beyond the protection from high-temperature stress. In Arabidopsis and other plant species, Hsps may also be induced under various stress conditions, including low temperature stress (Sabehat et al. 1998), oxidative stress (Desikan et al. 2001, Volkov et al. 2006), osmotic stress or desiccation (Sun et al. 2001, Liu et al. 2006, Swindell et al. 2007).

In plants, there are five main families of Hsps that are grouped according to their average molecular weights: Hsp100, Hsp90, Hsp70, the chaperonins Hsp60 and the small Hsp (sHsp) family (reviewed by Wang et al. 2004). Among them, the 90-kDa Hsp90 family differs from most other molecular chaperones in that their known 'client' proteins are signal transduction proteins, like transcription factors and kinases (Richter and Buchner 2001, Young et al. 2001), many of which are likely to participate in controlling plant growth and development (Chory and Wu 2001). Hsp90s are highly conserved across all eukaryotes and are considered essential for the vitality of cells. Besides their major role in the response to abiotic or biotic stress by managing protein folding or degradation (Lu et al. 2003, Liu et al. 2004, Chen et al. 2006, Samakovli et al. 2007, Jarosz and Lindquist 2010), Hsp90s are also involved in protein trafficking and cell cycle control (Wang et al. 2001). Recent studies have uncovered central roles of the Hsp90 chaperone system in plant development and evolution. By reducing their function, it was shown that they influence the Arabidopsis morphogenetic responses to environmental cues and buffer canonical development from destabilising effects of stochastic processes (Queitsch et al. 2002, Sangster et al. 2004, Samakovli et al. 2007). Thus, Hsp90 may also serve as an evolutionary-conserved buffering agent of phenotypic variation, possibly by suppressing the mutagenic activity of transposons (Specchia et al. 2010).

Although different *Hsp90* genes have been isolated from several annual herbaceous plants, the only well-characterised Hsp90 gene family so far remains that of the model plant *Arabidopsis thaliana*, which possesses seven members. AtHsp90.1 to AtHsp90.4 constitute the cytoplasmic subfamily (Milioni and Hatzopoulos 1997), AtHsp90.5 is localised in the chloroplast (Cao et al. 2003), and AtHsp90.6 is localised in the mitochondria (Prassinos et al. 2008), while AtHsp90.7 is an endoplasmic reticulum (ER)-resident protein (Ishiguro et al. 2002). In grapevine, research on Hsp90 or other Hsp families has been very scarce. In a pioneering study, Morrell et al. (1997a,b) reported the accumulation of Hsp70 proteins in leaves and dormant buds of Cabernet Sauvignon grapevines subjected to heat shock treatment. More recently, an Hsp90 gene was cloned from *Vitis pseduoreticulata* (Xu and Wang 2009), while Kobayashi et al. (2010) characterised four grapevine members of the sHsp family.

Given the significance of Hsp90s in plant stress response and canonical development, the scope of the present study was to characterise, for the first time, the grapevine Hsp90 family and to monitor the expression of different genes under thermal stress conditions. The regulation of gene expression was also assessed under combined heat and drought stress, as detrimental effects of thermal stress often interact with seasonal water deficit to accelerate plant strain (Cramer 2010). Present results point to sub-functionalisation among isoforms, while a heat-inducible cytosolic gene (*VvHsp90.1a*) may also play a role in tendril development.

Materials and methods

In silico *identification and analysis of grapevine Hsp90 sequences*

To identify putative *Hsp90* genes in grapevine, the genome sequence of the Pinot Noir clone PN40024 (Jaillon et al. 2007) was BLAST searched at the Genoscope Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), which uses the GAZE computational framework for the identification of protein-coding genes (Howe et al. 2002), and the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), which uses the Gnomon gene annotation program. Sequences originating from the Istituto Agrario San Michele all'Adige (IASMA) genome-sequencing project of the Pinot Noir clone ENTAV 115 (Velasco et al. 2007) were retrieved from NCBI. The chromosomal locations of genes were obtained using the BLAST-Like Alignment Tool (BLAT) server and additional physical localisation tools at the Genoscope website or by analysing the available information at the NCBI. Multiple sequence alignments were conducted using the ClustalW alignment tool of the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The exon–intron composition of putative *Vitis vinifera Hsp90* genes was automatically determined by the GAZE program at the Genoscope website or was derived from the European Nucleotide Archive at the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (http://www.ebi.ac.uk/ena/). Gene structure was further inspected through sequence comparisons with corresponding *V. vinifera* expressed sequence tags, when available, and alignments with Hsp90 sequences from other plant species. When necessary, the position of the intron was corrected according to the 5′ and 3′ splicing consensus sequences NN/GT and AG/NN (where N = any nucleotide), respectively. Subcellular localisation of the deduced polypeptides was predicted by WoLF PSORT (http://wolfpsort.org/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) software packages. Molecular weight of polypeptides was determined using a web-based software within the sequence manipulation suite (http://www.bioinformatics.org/sms/). Phylogenetic analysis was conducted using the neighbour-joining algorithm included in the MEGA 4.1 software package (Tamura et al. 2007). Multiple-aligned sequences corresponding to conserved regions, characteristic of the Hsp90 family, were determined by searching the BLOCKS database, version 14.2 (http://bioinformatics.weizmann.ac.il/blocks/). The Hsp90 gene family signature was detected by scanning sequences against PROSITE patterns and profiles, through the ExPASy proteomics server (http://au.expasy.org/prosite/).

Plant material and treatments

All plant material used for RNA extraction, except for roots, was obtained from grapevines (*V. vinifera* L. 'Agiorgitiko') grafted on R-110 rootstock (*V. rupestris* × *V. berlandieri*). Plants were grown in 4.7-L pots containing a mixture of soil : peat : perlite (1:1:1) in a climate chamber (Snijders Scientific, Tilburg, The Netherlands) under a 16/8-h photoperiod (light intensity of 240 $\mu E.m^2/s^2$), 65% of relative humidity and at a constant temperature of 23°C, unless otherwise stated. Irrigation was performed twice a week to sustain a midday leaf water potential of about −0.5 Mpa, while for the application of drought stress, plants were subjected to water shortage for 21 days, until the leaf water potential reached

approximately −1.5 Mpa. Leaf water potential was measured with a pressure chamber instrument (Model 615, PMS Instrument Company, Albany, Oregon, USA). Roots were obtained from *in vitro*-grown micropropagated Agiorgitiko plants, regenerated through single-node cuttings without the addition of plant growth regulators, as described previously (Banilas and Korkas 2007). For heat shock treatment, plants were transferred to 30, 37 or 45°C for different time periods (15–120 min) as indicated in the Figures. Samples were immediately frozen in liquid nitrogen and stored at −80°C for later RNA extraction.

RNA extraction and RT reactions

Total RNA was isolated from shoot apical meristems, young expanding leaves (from second to third node), mature leaves (basal node), roots, young shoots (first and second internodes) and developing tendrils. A previously described method for *V. vinifera* genomic DNA extraction (Siles et al. 2000) was slightly modified and adapted for RNA isolation. The extraction buffer contained 100 mM tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 2.0% (w/v) CTAB, 2% (w/v) poly(vinylpolypyrrolidone) (PVPP) and 0.2% (v/v) β-mercaptoethanol. Briefly, approximately 50 mg of tissue was ground under liquid nitrogen and transferred into a 1.5-mL microcentrifuge tube containing 500 µL of the extraction buffer. The mixture was shaken vigorously and incubated at 65°C for 30 min before centrifugation at 16 000 × *g* for 10 min. The supernatant was then extracted twice with chloroform : isoamyl alcohol (24:1), and the nucleic acids were precipitated by the addition of 0.5 volume of 5 M NaCl, followed by 1 volume of isopropanol and centrifugation at 16 000 × *g* for 10 min. The resulting pellet was washed with cold 75% (v/v) ethanol and dissolved in ddH_20. Co-precipitated DNA was eliminated by DNase I treatment (RQ1 RNase-free DNase, Promega). The RNA concentration was determined spectrophotometrically and verified by ethidium bromide staining of 1% (w/v) agarose gel. About 0.5 µg of total RNA was used as template in first strand cDNA synthesis using Superscript™ II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, California, USA), according to the manufacturer's protocol. The first-strand cDNA was primed off by the poly-A tail with the primer T17XHO (5′-GTCGACCTCGAGTTTTTTTTTTTTTTTT-3′). Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

The sequences of gene-specific primers used in RT-PCR experiments (Table 1) were designed using Primer3Plus (Untergasser et al. 2007). The specificity of primers was checked by NCBI BLAST searches, limited to the grapevine genome and selecting the 'short-queries' parameter for short-input sequences and verified by direct sequencing of the PCR products and inspection of the sequence chromatograms. RNA was extracted from leaves (from second to fourth node) and used in RT reactions as described above. All amplifications were performed in a MJ Mini thermal cycler (Bio-Rad, Hercules, California, USA) with equal amounts of templates together with 200 µM of each dNTP, 20 pmol of each primer, 1 U of DNA polymerase (DyNAzyme EXT, Finnzymes) and 1 × PCR buffer (provided by the manufacturer of the enzyme) in a 50-µL reaction volume. After an initial denaturation step at 94°C for 90 s, a different number of cycles were run, each at 94°C for 30 s, 60 or 64°C (according to the primer set) for 30 s and 72°C for 30 s, before a final extension at 72°C for 5 min. The number of PCR cycles was determined for each gene to optimise reproducibility and to ensure that amplification occurred within a linear range. Three independent PCR reactions were performed per gene, resulting to reproducible results. The grapevine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (GenBank accession No XM_002263109) was used as control. RT-PCR reactions (10 µL) were run on 1% (w/v) agarose gel, stained using ethidium bromide and photographed using Gel Doc (Bio-Rad) imaging system. PCR products were gel-extracted (QIAquick Gel Extraction Kit, Qiagen GmbH, Hilden, Germany) and directly sequenced (Macrogen Inc., Seoul, Korea) to confirm their identity.

Results

In silico *identification and characterisation of* V. vinifera *Hsp90 sequences*

The whole grapevine genome sequence of the genotype PN40024, published by Genoscope, was initially searched for *Hsp90* genes. After excluding predicted genes with no consistent homology to Hsp90s or with an incomplete open-reading frame, three putative *Hsp90s* were retained for further analysis. Their deduced amino acid sequences (GenBank accession Nos. CBI27411, CBI28422 and CBI19885) showed very high homologies, throughout their entireties, to the Arabidopsis organellar Hsp90 proteins AtHsp90.5, AtHsp90.6 and AtHsp90.7, respectively. As an exception, the CBI28422 sequence was predicted to have an atypical N-terminal extension of 72 aa, in respect to AtHsp90.6 and other homologous plant proteins. Inspection of its gene sequence (GSVIVT01030056001) showed that the first intron is unlikely to be spliced at the TT-TA sites, according to the canonical GT-AG splice-site junctions. After multiple sequence alignments and sequence comparison with its counterpart from the Pinot Noir clone ENTAV 115 (GenBank accession No CAN79988), the start codon for translation was manually corrected to be within the second exon, i.e. 135 nucleotides downstream from the 3′ splice site of the first intron. Based on different algorithms, it was estimated that the proteins CBI27411, CBI28422c (corrected as above) and CBI19885 are located to

Table 1. Primers used for semi-quantitative RT-PCR analysis of *VvHsp90s* and the *GAPDH* reference gene.

Gene abbreviation	Sequence of primer pair (5′–3′)	PCR product size (bp)
VvHsp90.1a	aactgagaaagagatcagtgatgatg/cagtgatgtagtagatgtccttctgg	758
VvHsp90.2a	ggaaggatctagaaaccctagaatg/caagctctcaaatcttatcttgtcc	268
VvHsp90.5	aatgaatcccaaaacaaagaacat/cttaatagtggtgttatgcgcttg	342
VvHsp90.6	acctgaagtcatacaaggagaagaat/ggagtgaaaccactagaaattaaagc	433
VvHsp90.7	aggttattttctttactgacccagtg/tcctttactactctttcctgaagctc	400
GAPDH	gccactctttagcttgtttctctc/cgttaactccaacaacgaacatag	488

Figure 1. Sequence alignment of deduced Arabidopsis and grapevine Hsp90 proteins. Identical or similar amino acids are shaded black or grey, respectively. The positions of conserved functional domains are indicted above the aligned sequences. The most highly conserved regions, characteristic of the Hsp90 family, were determined by searching the BLOCKS database. The HATPase-c conserved signature sequence of Hsp90s (dotted line) was detected by scanning the sequences against PROSITE patterns and profiles, through the ExPASy proteomics server. The Hsp90 signature motif 'Y-x-[NQHD]-[KHR]-[DE]-[IVA]-F-[LM]-R-[ED]', close the N-terminal region, is bounded by a rectangle drawn by dashed lines.

chloroplast, mitochondrion and ER, respectively. The latter polypeptide also contains the C-terminal 'KDEL' motif, which marks proteins for ER retention (Munro and Pelham 1987). Multiple sequence alignment (Figure 1) and phylogenetic analysis (Figure 2) verified that the above proteins represent the grapevine orthologs of the Arabidopsis organelle-specific Hsp90 members. To be consistent with the nomenclature scheme of Arabidopsis, these grapevine proteins are referred to herein as VvHsp90.5, VvHsp90.6 and VvHsp90.7, respectively (Table 2).

Three additional putative VvHsp90 members were detected through BLAST searches at NCBI. Their deduced amino acid sequences (NCBI accession Nos XP_002274022, XP_002278894 and XP_002273244) are highly similar to each other and also to the cytosolic members of the Arabidopsis Hsp90 family. Those genes were not annotated at Genoscope, probably due to differences in gene prediction methods, but they were all identified in the IASMA genome-sequencing project of the clone ENTAV 115 (Velasco et al. 2007). The gene encoding XP_002274022, contained in ChrUn_random (ultracontigs whose physical positions on specific chromosomes have not been defined), was assigned to chromosome 16 by analysing the available information at NCBI from the IASMA sequencing project. BLAST searches in the IASMA protein database made it possible to identify an additional putative VvHsp90 sequence (GenBank accession No CAN62488), for which no counterpart in the genome of the PN40024 clone could be identified, according to its distinct chromosomal location (Chr 10; Table 2). Therefore, it was retained for further analysis, as a putative unique *VvHsp90* gene. Subcellular localisation of the above four polypeptides was predicted to reside in the cytoplasm. Moreover, they all have the C-terminal pentapeptide 'MEEVD', which is a diagnostic sequence motif of cytoplasmic Hsp90s in both plants and animals (Krishna and Gloor 2001). Multiple-sequence alignment (Figure 1) and phylogenetic analysis (Figure 2) revealed that two of them are highly similar to each other and are clearly grouped with AtHsp90.1 (Table 3). Therefore, they were designated as VvHsp90.1a and VvHsp90.1b. The other two proteins are also closely related to each other (Table 3), but not placed within the group of Hsp90.1 or close to any particular Arabidopsis member (Figure 2). Therefore, they were designated, in ascending order, as VvHsp90.2a and VvHsp90.2b (Table 2).

All seven VvHsp90s identified possess the Hsps90 family signature motif 'Y-x-[NQHD]-[KHR]-[DE]-[IVA]-F-[LM]-R-[ED]' close to the N-terminal region (Lindquist and Craig 1988) (Figure 1) and share significant sequence homology with each other (Table 3), especially the cytoplasmic members. The gene structure organisation of different genes is very similar to their Arabidopsis counterparts. Both *VvHsp90.1a* and *VvHsp90.1b* have four exons, *VvHsp90.2a* and *VvHsp90.2b* share a similar three-exon structure, while genes encoding the organelle-specific VvHsp90s have multiple exons (Figure 3).

Tissue-specific expression of VvHsp90 genes under heat shock

Gene-specific primers were designed for the organellar members (*VvHsp90.5-VvHsp90.7*) and for one gene from each cytosolic group, i.e. *VvHsp90.1a* and *VvHsp90.2a* (Table 2). RT-PCR employing RNA extracted from young leaves under heat shock conditions (45°C for 30 min) resulted into the amplification of a single band per gene of the expected size and sequence (data not shown). Those primers were further used to determine the steady-state expression levels of *VvHsp90* genes through semi-quantitative RT-PCR analysis in various vegetative tissues/organs of grapevines grown at 23°C (control) or after heat shock treatment at 45°C for 30 min (Figure 4). The two cytosolic genes (*VvHsp90.1a* and *VvHsp90.2a*) showed quite distinct expression patterns. *VvHsp90.1a* was strongly up-regulated after heat shock in all organs tested, while no transcripts were detectable under control conditions, even after 38 PCR cycles. As a noteworthy exception, it was expressed at relatively high levels in tendrils of control plants, although transcripts increased substantially after heat shock. As opposed, the expression patterns of *VvHsp90.2a* in different tissues/organs resembled those of the housekeeping *GAPDH* gene. Under control conditions, the organellar genes (*VvHsp90.5-VvHsp90.7*) were expressed in all tissues/organs at comparable levels, except for *VvHsp90.7* in roots and shoots, where transcripts were at relatively low levels. Heat shock treatment did not induce the expression of the organellar *VvHsp90* genes significantly. Hence, among the *VvHsp90* genes analysed, only *VvHsp90.1a* performed as a *bona fide* heat-inducible gene under the particular heat-stress conditions applied.

Temperature- and time-dependent expression of VvHsp90s

Because a heat shock treatment at a relatively high temperature point and constant time period resulted into the apparent up-regulation of only *VvHsp90.1a*, we asked how different *VvHsp90s* would be regulated at lower, albeit elevated, temperatures and/or after a longer duration under stress. In this respect, gene expression was analysed in leaves from heat-treated plants at 30, 37 or 45°C for 30, 60 or 120 min. As it is shown in Figure 5, the expression pattern of *VvHsp90.1a* varied in respect the severity and the duration of the heat stress applied. While its transcripts were barely detectable after 120 min at 30°C, a sharp up-regulation was observed as early as 30 min at 37°C. Thereafter, transcripts decreased slightly but remained at elevated levels. *VvHsp90.1a* up-regulation was also apparent after 30 min at 45°C, but the mRNA levels were higher after 60 or 120 min. As opposed to *VvHsp90.1a*, the expression of *VvHsp90.2a* was not induced after any tested heat shock treatments, and transcripts remained at low levels. Similarly, the organellar *VvHsp90* genes were not induced by different heat-stress treatments applied, except for some mild up-regulation of *VvHsp90.7* after 60 min at 37 or 45°C (Figure 5).

Combined effect of drought and heat stress on VvHsp90 expression

Because grapevines in nature often encounter a combined effect of high temperature and drought stress, we asked how this combination could affect the expression of different *VvHsp90s*. Given the severity of such a combined stress treatment, sampling of developing leaves was started as early as 15 min after the application of heat shock and lasted for a total of 60 min. For comparison reasons, samples of well-watered plants were also included in the analysis. As it is shown in Figure 6, when drought

was applied as the sole stress, the steady-state expression levels of different *VvHsp90s* remained almost unchanged. In well-watered plants, heat shock induced the expression of *VvHsp90.1a* within 15 min. In the respective drought-stressed plants, however, up-regulation of *VvHsp90.1a* was delayed, and transcripts reached high levels after 30 min. The combined heat and drought stress did not induce the expression of the other *VvHps90s*, and their expression patterns resembled the ones detected in the well-watered plants. In accordance to previous observations (Figure 5), transcripts of *VvHsp90.7* increased slightly after 60 min of heat shock treatment, but only in the well-watered plants.

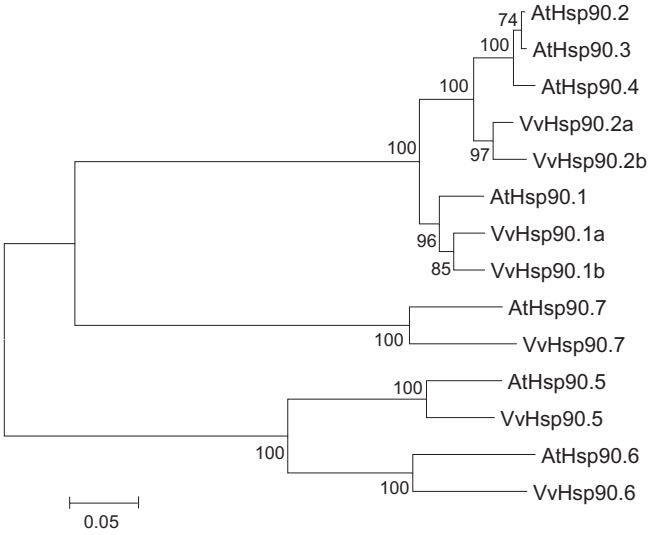

Figure 2. Phylogenetic relationships among *Vitis vinifera* (VvHsp90) and *Arabidopsis thaliana* (AtHsp90) heat shock protein 90 isoforms. The NCBI/GenBank accession Nos for the grapevine proteins are shown in Table 2. The accession Nos for AtHsp90.1–AtHsp90.7 proteins are BAA98082, BAB09285, BAB09283, BAB09282, AAD32922, AAF13098 and CAB45054, respectively. The tree was constructed according to the neighbour-joining algorithm. Numbers at branch points are bootstrap percentages derived from 1000 replicates.

Discussion

The availability of the complete grapevine genome sequence (Jaillon et al. 2007, Velasco et al. 2007) provides an opportunity for the identification and characterisation of major gene families in *V. vinifera* (Martinez-Zapater et al. 2010). Following an integrated approach to detect Hsp90s in grape, seven unique genes were identified. Few additional partial sequences with homologies to Hsp90s were detected, which most probably represent pseudogenes. The total number of genes identified is equal to that of the Arabidopsis gene family. In Arabidopsis, there are four cytoplasmic isoforms. Among them, *AtHsp90.1* is a true Hsp90 heat-inducible molecular chaperone, while the *AtHsp90.2–4* genes are constitutively expressed and only mildly induced by heat shock (Takahashi et al. 1992, Yabe et al. 1994, Milioni and Hatzopoulos 1997). *AtHsp90.2–4* genes are closely related to each other, both in sequence similarity and in physical position, being immediately adjacent on the same chromosome and sharing the same intron/exon structure (Milioni and Hatzopoulos 1997). This suggests that they might have originated via quite recent gene duplication events and are thus considered functionally redundant (Krishna and Gloor 2001).

The present study uncovered a total of four genes encoding cytoplasmic Hsp90s in *V. vinifera*. However, in contrast to Arabidopsis, cytoplasmic *VvHsp90s* are clearly divided into two phylogenetically diverse groups of two genes per group (*VvHsp90.1a-b* and *VvHsp90.2a-b*). Because the four cytosolic *VvHsp90s* are dispersed to different chromosomes, it is plausible to hypothesise that they are relatively old gene copies because young gene duplicates tend to lie adjacent to each other on the same chromosome (Chen et al. 2005, Rizzon et al. 2006). As the cytosolic isoforms within each group are highly similar to each other (97–98% similarity), they likely represent functionally redundant paralogs. However, we can not exclude the possibility that these genes may have different transcriptional characteristics and/or roles.

Table 2. Genome position and annotation reference for *VvHsp90* genes, as given on the Genoscope website or at NCBI/GenBank, their deduced polypeptides and predicted subcellular localisation.

Gene name	Locus tag/gene ID	NCBI accession No	Protein length (aa)	MW (kDa)	Subcellular localisation	Chromosome location
VvHsp90.1a	LOC100257736	XP_002274022	704	80.84	Cytoplasm	Chr 16 from 18053156 to 21785637 strand +
VvHsp90.1b	LOC100265661	XP_002278894	703	80.86	Cytoplasm	Chr 2 from 220430 to 223397 strand –
VvHsp90.2a	VITISV_029391	CAN62488	699	80.05	Cytoplasm	Chr 10 from 17924 to 21484 strand –
VvHsp90.2b	LOC100261759	XP_002273244	704	80.86	Cytoplasm	Chr 19 from 9148553 to 9151792 strand –
VvHsp90.5	GSVIVT01010120001†	CBI27411	750	85.83	Chloroplast	Chr 1 from 15794280 to 15805183 strand +
VvHsp90.6	GSVIVT01030056001	CBI28422c‡	799	90.72	Mitochondrion	Chr 12 from 9294601 to 9304381 strand +
VvHsp90.7	GSVIVT0100993100	CBI19885	818	93.23	Endoplasmic reticulum	Chr18 from 12501749 to 12507830 strand –

†GSVIVT numbers represent the region of Genoscope annotation and the identifier from the Grape Genome Browser. ‡Corrected as shown in Figure 1 (see text for details). NCBI, National Centre for Biotechnology Information.

Table 3. Percentages of amino acid identity (standard font) and similarity (italics) between the grapevine Hsp90 proteins.

–	VvHsp90.1a	VvHsp90.1b	VvHsp90.2a	VvHsp90.2b	VvHsp90.5	VvHsp90.6	VvHsp90.7
VvHsp90.1a	–	94	86	85	42	39	43
VvHsp90.1b	*98*	–	87	86	41	39	44
VvHsp90.2a	*92*	*93*	–	94	41	38	42
VvHsp90.2b	*92*	*92*	*97*	–	40	37	42
VvHsp90.5	*61*	*62*	*61*	*61*	–	64	38
VvHsp90.6	*58*	*59*	*58*	*58*	*76*	–	38
VvHsp90.7	*60*	*60*	*60*	*60*	*58*	*57*	–

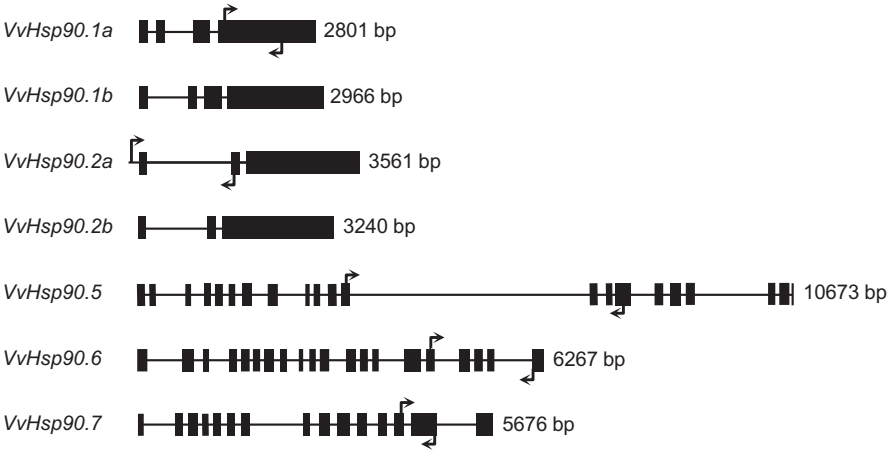

Figure 3. Exon–intron structures of *V. vinifera Hsp90* genes. The exons are represented as gray boxes, and the introns are represented as lines. Total gene lengths, from the predicted translation initiation codon (left exon) till the stop codon (right exon), are indicated to the right of each gene. Arrows indicate the approximate target sites for gene-specific primers (Table 1). In the case of *VvHsp90.2a*, the forward primer was designed to anneal at the 5′-untranslated region (115-nt upstream the translation initiation codon).

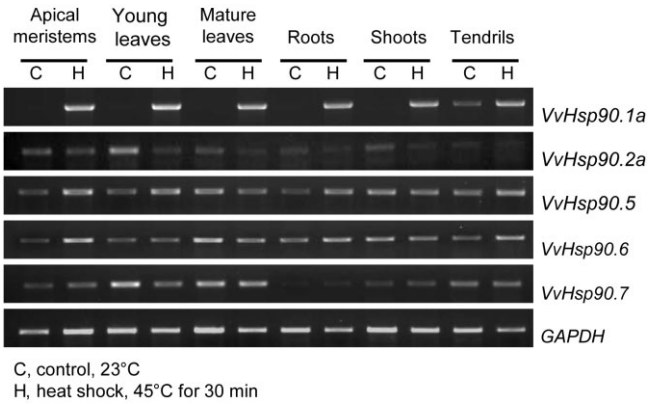

Figure 4. Semi-quantitative RT-PCR analysis of *VvHsp90s* in various vegetative tissues/organs and tendrils of grapevines grown under control conditions (C) or after heat shock (H). To ensure equal amounts of template, the grapevine *GAPDH* gene was used as a reference gene.

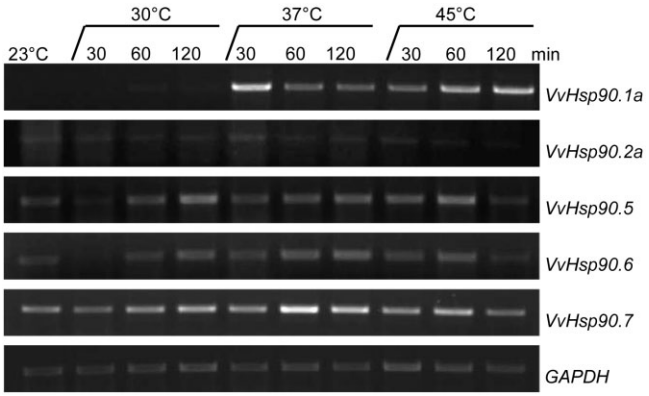

Figure 5. Transcriptional analysis of *VvHsp90s* in leaves of grapevines grown at 23°C and then subjected to mild, modest or severe heat shock (30–45°C) for different time periods (30–120 min). *GAPDH* was included as a reference gene.

The organelle-specific VvHsp90 proteins share high-sequence similarities with their Arabidopsis orthologs. Moreover, they are also represented by single-copy genes and are composed of multiple introns/exons of similar or equal numbers to their Arabidopsis counterparts. Those similarities support previous suggestion that the organellar *Hsp90s* were produced very early during the evolution of eukaryotes (Chen et al. 2006), definitely before the radiation of the Eurosids into Eurosids I (*V. vinifera*) and Eurosids II (Arabidopsis) (Jaillon et al. 2007), following concerted evolution thereafter.

The induction of *Hsps* is known to be regulated mainly at the transcriptional level (Morimoto 1993). Members of the prokaryotic family are expressed at very low levels under favourable conditions, but they are strongly induced by heat shock or other environmental stress (Mason et al. 1999). As opposed, *Hsp90s* in mammals are constitutively expressed and only modestly up-regulated under heat shock (Buchner 1999). In plants, the expression of *Hsp90s* was shown to be developmentally regulated in different organs (Reddy et al. 1998, Haralampidis et al. 2002), determining developmental plasticity of the plant body (Samakovli et al. 2007). In Arabidopsis, *AtHsp90.1* is highly expressed under heat shock, while under control conditions, transcripts may be detected only in roots (Yabe et al. 1994). Similarly in grapevine, expression of *VvHsp90.1a* was apparent in different vegetative organs after

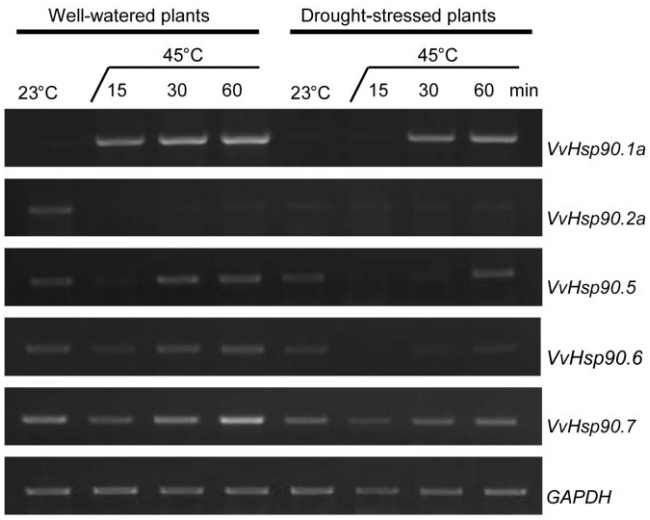

Figure 6. Transcript profiles of *VvHsp90s* in leaves of well-watered or drought-stressed grapevines grown at 23°C and then subjected to severe heat shock (45°C) for different time periods (15–60 min). *GAPDH* was used as a reference gene.

heat shock but undetectable under favourable conditions. As an interesting exception, transcripts of *VvHsp90.1a* were also apparent in tendrils under non-stress conditions. Grapevine has a different pattern of organ development from annual herbaceous plants like Arabidopsis (Mullins et al. 1992, Boss et al. 2003, Carmona et al. 2008). The expression of *VvHsp90.1a* in tendrils in the absence of stress could mark specific developmental differences between tendrils and vegetative organs. Given that tendrils are considered modified reproductive structures in *Vitaceae*, present results underline the importance for further *Hsp90* transcriptional analysis during grape reproductive organ development.

Although our knowledge on Hsp90 mode of function during plant development is still limited (Samakovli et al. 2007), it has been proposed that Hsp90 activity is required for brassinosteroid-mediated signal transduction, similarly to the steroid-dependent signalling in other organisms (Amzallag and Goloubinoff 2003, Sangster and Queitsch 2005). Because brassinosteroids were recently associated with grape berry ripening (Symons et al. 2006), the investigation of possible role(s) of Hsp90 machinery in signal-transduction pathways during grape berry development is thus of particular interest.

Transcriptional analysis revealed that *VvHsp90.1a* is differentially regulated in respect to the severity of the heat stress. As opposed, *VvHsp90.2a* was weakly and constitutively expressed. *VvHsp90.1a* mRNA levels increased with time under severe heat shock (45°C), while under moderate stress conditions (37°C), transcripts declined after an initial peak (Figure 5). Similar results were also obtained in potato (*Solanum tuberosum*), where the synthesis of Hsps was stronger and/or continued for a longer time period upon heat stress at 40 than 35°C (Ahn et al. 2004), suggesting that plants not only sense shifts in temperature but may also monitor the magnitude of heat stress.

The genes-encoding organellar VvHsp90s were constitutively expressed in various organs under non-stress conditions. This may not be surprising considering that Hsp90s are quite abundant proteins, normally accounting for 1–2% of total proteins in cells, to maintain proper cell function and homeostasis. Only a mild transient up-regulation of *VvHsp90.7* was observed after 60 min at 37 or, to a lesser extent, at 45°C (Figure 5). In contrast to the well-characterised cytosolic Hsp90s, expression characteristics of ER *Hsp90s* are still largely unexplored. However, accumulating data suggest that ER-targeted Hsp90s may be synthesised upon heat stress, probably triggered by the accumulation of misfolded proteins (Meunier et al. 2002, Cha et al. 2009). Present results are also in general agreement with a previous transcriptional analysis of an ER *Hsp90* gene from rice, where heat shock caused gene up-regulation after 30 min, but soon after transcripts declined (Liu et al. 2006).

In the vineyard, thermal stress is often accompanied with other types of abiotic or biotic stress. Among them, seasonal drought is often the most important constraint to grapevine growth. It was previously shown that several *Hsps*, including a cytosolic *Hsp90*, were induced in tobacco plants subjected to combined drought and heat stress (Rizhsky et al. 2002). Furthermore, the high-osmolarity glycerol mitogen-activated protein kinase pathway in *Saccharomyces cerevisiae*, which is activated by osmotic stress, may be also activated by heat stress, with a regulatory role of Hsp90 machinery in downstream signalling pathways (Winkler et al. 2002, Millson et al. 2005). However, the physiological roles of Hsp90 chaperone complexes in plants are largely unknown, and species-specific differences may also exist. The severe drought stress applied in this study did not induce the expression of *VvHsp90s* examined (Figure 6). As opposed, a mild down-regulation of *VvHsp90.2a* was detected. In Arabidopsis, it was recently shown that the overexpression of *AtHsp90.2*, *AtHsp90.5* or *AtHsp90.7* reduced plant tolerance to drought stress, assuming that excessive Hsp90 may cause a general stress itself, either by challenging the organellar import/export system or by disturbing the native protein homeostasis, which is necessary for normal osmotic stress resistance (Song et al. 2009).

Drought stress may operate synergistically with heat stress causing much greater detrimental effects on plants than when applied individually (Rizhsky et al. 2004, Mittler 2006, Edwards et al. 2011). When drought was combined with heat stress, the expression patterns of different *VvHsp90s* remained rather unchanged; nevertheless, the up-regulation of *VvHsp901a* was delayed. It seems likely that in grapevine, like in other plants, severe drought stress preferentially activates other molecular responses, including the synthesis of various sHsps and Hsp70s, the up-regulation of antioxidants and/or the accumulation of compatible solutes (Cushman and Bohnert 2000, Xiong and Zhu 2002).

Conclusions

The whole grape genome sequence was searched, and seven *Hsp90* genes were identified. Expression characteristics of different *VvHsp90s* resembled those of their homologous genes in Arabidopsis or other plant species. Transcriptional analysis of selected genes uncovered that the *Hsp90.1a* is a *bona fide* heat-inducible gene. A role of *VvHsp90.1a* might also be ascribed to tendril development, highlighting a promising implication of VvHps90 in the development of reproductive organs. Expression patterns of different *VvHsp90s* varied either under normal or stress conditions, pointing to possible isoform sub-functionalisation. Given the importance of this gene family in plants, present results may ameliorate further research to better understand the molecular mechanisms of grapevine response to environmental constraints, like various types of abiotic or biotic stress, and to ascribe roles of Hsp90 machinery in grapevine development and morphological evolution.

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