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African Journal of Biotechnology Vol. 9 (7), pp. 927-949, 15 February, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

*Review*

# An overview on the small heat shock proteins

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Accepted 7 October, 2009

**In the last 25 years, a huge amount of literature has been accumulated describing the cell's response to different kinds of environmental stress conditions, such as high temperatures, altered pH, exposure of the cell to toxins, starvation, oxygen, and water deprivation, among others. Heat shock proteins (HSPs) are one of the main expressed products of the cell in response to stresses. HSPs can be classified into six structurally conserved classes according to their molecular weight namely, HSP100, HSP90, HSP70, HSP60, small heat shock proteins (sHSPs) and ubiquitin (8.5 kDa). In eukaryotes, different heat shock genes are expressed uncoordinatedly, whereas in prokaryote, heat shock genes form a regulon and appear simultaneously. sHSPs are associated with nuclei, cytoskeleton and membranes. They bind partially to denatured proteins, preventing irreversible protein aggregation during stress. In animals, only one sHSP gene has been located in yeast cells, ten in mammalian, two in birds and four genes have been found in *Drosophila*. However, in plants more than 20 sHSPs have been reported and they can be divided into 6 classes, of which, 3 classes (CI, CII and CIII) are in the cytosole or in the nucleus and the other three (CIV, CV and CVI) in the plastids, endoplasmic reticulum and mitochondria. Mitochondrial and chloroplast sHSPs protect electron transport chain. During development in animals, sHSP genes are normally regulated at late neurula and early tailbud stage and in plants during pollen development, seed maturation, seed imbibition and germination. Transcriptional regulation of sHSPs depends on particular activation of heat shock factors (HSF) which recognize the highly conserved heat-shock elements (HSEs). After the heat stress has been released, the sHSPs are quite stable, suggesting that sHSPs may be important for recovery as well.**

**Key words:** Small heat shock proteins, localization, structural dynamics, function, molecular evolution.

## INTRODUCTION

Heat shock proteins (HSPs) are a class of proteins whose expression is increase when cells are exposed to elevated temperatures or other stress. This increase in expression is transcriptionally regulated primarily by heat shock factor (HSF). HSPs are found in all living organisms, from bacteria to humans. HSPs are involved in the folding of denatured proteins. High temperatures and other stresses, such as altered pH and oxygen deprivation, make it more difficult for proteins to form their proper structures and cause some already structured proteins to unfold. Increased expression of HSPs is mediated at multiple levels: mRNA synthesis, mRNA stability, and translation efficiency. Heat-shock proteins are named according to their molecular weight, such as HSP60, HSP70, HSP90, HSP100, and the small heat-shock proteins (sHSPs) (Schlesinger, 1990).

In prokaryote, the events regulating heat shock gene expression vary in quite a few respects. Firstly, unlike the eukaryotes where different heat shock genes are expressed uncoordinatedly, heat shock genes in the prokaryote form a regulon and appear simultaneously. Secondly, the heat shock transcription factor is an isomer of the $\sigma$ subunit, the regulatory element in the bacterial RNA polymerase. This $\sigma$ factor exists at low levels under normal growth conditions, but its levels rise quickly after heat shock due to much slower degradation of the protein, enhanced translation of its mRNA, and increased transcription of the gene. The mRNAs from heat shock genes

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have structures that permit for their selective translation in a stressed cell. These include lack of intron(s), regions in the 5'-untranslated regions conferring translational efficiency, and regions in the 3'-untranslated segments providing for increased stability (Schlesinger, 1990).

In Eukaryotes, the temperature at which maximum HSPs can synthesize themselves, changes according to the species. Plants synthesize HSPs proportionally with the severity of heat shock until the greatest level to tackle the shock. HSP synthesis is completely induced for survival with maximum activation of other protection mechanisms at near deadly temperatures. However, plants probably synthesize middle level HSPs at mild heat stress conditions at first, but if the heat stress continues, then more synthesis of HSPs is carried out (Ahn et al., 2004). It is known that rapid heat hardening can be elicited by a brief exposure of cells to sub-lethal high temperature, which in turn provides protection from subsequent and more severe temperatures (Schlesinger, 1990).

## HEAT SHOCK PROTEIN FAMILIES

Heat shock proteins can be classified into six structurally conserved distinct classes namely HSP100, HSP90, HSP70, HSP60 (Chaperonins), approximately 17-30 kDa molecular weight small HSPs (sHSPs) and ubiquitin (8.5 kDa) (Vierling, 1997, Waters et al., 1996). High molecular weight HSPs show high level of sequence similarity but differ in terms of their specific function even among HSP homolog, which belongs to the same family and functioning in different cellular compartments (Vierling, 1997).

**HSP100 family:** Members of this family are up-regulated by environmental stress. Proteins of this family generally function to protect protein denaturation and protein aggregation (Wang et al., 2004). It was reported that over expression of HSP101 in *Arabidopsis* has positive effect on growth during recovery period (Vinocur and Altman, 2005). It was recently found that HSP101 homologue in *Arabidopsis* is involved in plastid differentiation, mediating internal thylakoid membrane formation and conferring thermotolerance to chloroplasts during heat stress (Lee et al., 2007).

**HSP90 family:** This family consists of proteins which have highly conserved amino acid sequence. Proteins of this family are especially rich in cytoplasmic phosphoproteins (Nover et al., 1989). The major role of HSP90 is to manage protein folding. But it can also play a key role in signal transduction networks, cell cycle control, protein degradation and protein trafficking. In addition, it might also play a role in morphological evolution and stress adaptation in *Drosphila* and *Arabidopsis*. It is among the most abundant proteins in cells (1-2% of total cellular protein). Although, HSP90 chaperones are constitutively expressed in most organisms, their expressions increase in response to stress in both prokaryotes and eukaryotes. Decreasing the level of functional HSP90 in *Drosophila* by genetic mutation or by treatment with an HSP90 inhibitor causes developmental abnormalities and morphological changes (Wang et al., 2004).

**HSP70 family:** Members of the HSP70 family were found to be highly conserved through the process of evolution. In addition, though strongly inducible by heat shock and other forms of cellular stresses, constitutively expressed HSP70s have essential functions under no stress conditions. In eukaryotes, homologs of these proteins have been found within mitochondria, cytoplasm, nucleus, endoplasmic reticulum and chloroplasts. These homologs accumulate in the nucleus after heat shock exposure (Hendrick and Hartl, 1993). Organellar HSPs have specific amino terminal target sequences, which provide HSP70s to translocate appropriate membrane sites. Some members of HSP70 family are phosphorrylated and/or methylated in vertebrates, yeast, bacteria and plants (Nover et al., 1989). It has been reported that *Arabidopsis* genome has at least 18 genes coding proteins of HSP70 family. Over expression of this gene induces thermal tolerance and increase in resistance to environmental stresses (Wang et al., 2004).

**HSP60 Family:** This family of highly conserved proteins of approximately 60 kDa is a chaperone. HSP60 helps in protein folding and subunit assembly. Their prokaryotic and eukaryotic members are dimeric and have phosphorylated isoforms. Members of this family are 14-subunit oligomers and they have been found in bacterial cytoplasm, while members of their stress-inducible and non-inducible members are present in bacterial cytosol and in the inner space of mitochondria and chloroplast. However, they have not been detected in endoplasmic reticulum and cytoplasm. Chloroplast chaperonin is required for the assembly of ribulose bisphosphate carboxylase/oxygenase (rubisco); hence its name was coined as “Rubisco binding protein” (Hendrick and Hartl 1993).

**Small heat shock proteins (sHSPs):** Small heat shock proteins (sHSPs) have been the Cinderella of the molecular chaperone world (Van den Ussel et al., 1999). Small heat shock proteins are associated with nuclei, cytoskeleton and membranes and bind partially denatured proteins, thereby preventing irreversible protein aggregation during stress (Sun and MacRae, 2005a). In somatic tissues of plants, sHSPs are the most abundant group of HSPs while 17-30 kDa are unique to higher plants. The abundance and heterogeneity of sHSPs suggest that they may have unique physiological functions (Vierling and Nguyen, 1992). Mammalian and yeast cells have only one sHSPs located in cytosol while four genes are found in *Drosophila* (Lindquist and Craig, 1988).

Plants, however, usually produce more than 20 HSPs and they are often the most abundant and stress responsive group of HSPs in plants (Heckathorn et al., 1999). Heterogeneity of sHSPs is unique to plants and with the exception of mitochondria localized sHSPs in animal cells, plants are the only eukaryotes in which organelle localized sHSPs have been described (Waters et al., 1996).

The sHSPs encoded by one gene family are similar to each other even in different plant species. The sequence similarity can be up to 93% and identity up to 85% (Vierling, 1991). However, sHSPs of one plant species belonging to different families show very low sequence similarity (50-75%), and identity usually below 50%. This applies not only for the comparisons of sHSPs between divergent species, but also for comparisons between different classes of plant sHSPs (Waters et al., 1996). Plant sHSPs are all encoded by nuclear genes and are divided into 6 classes. Of these, 3 classes (CI, CII and CIII) are localized in the cytosole or in the nucleus and the remaining three (CIV, CV and CVI) in the plastids, the endoplasmic reticulum and the mitochondria. All HSPs share a conserved 90 amino acid carboxyl-terminal domain called the α-crystalline domain or heat shock domain. The α-crystallin domain contains several beta-strands organized into two beta-sheets responsible for dimer formation; the basic building block of most sHSPs. The amino-terminal extension modulates oligomerization, subunit dynamics and substrate binding, whereas the flexible carboxy-terminal extension promotes solubility, chaperoning and oligomerization. The latter is by inter-subunit linkage (Sun and MacRae, 2005a). This domain distinguishes sHSPs from other small proteins induced by heat and might have an important role in chaperon activity (Waters et al., 1996).

The cytosolic class I small heat shock proteins (sHSP-CI) represent the most abundant sHSP in plants (Chang et al., 2001). Guan et al. (2004) reported the characterization and expression profile of nine members of the sHSP-CI gene family in rice, of which *Os*HSP16.9A, *Os*HSP16.9B, *Os*HSP16.9C, *Os*HSP16.9D and *Os*HSP 17.9B are clustered on chromosome 1, and *Os*HSP17.3, *Os*HSP17.7, *Os*HSP17.9A and *Os*HSP18.0 are clustered on chromosome 3 (Guan, et al., 1998). *Os*HSP17.3 and *Os*HSP18.0 are linked by a 356-bp putative bi-directional promoter. All sHSP-CI genes except *Os*HSP17.9B were induced strongly after a 2-h heat shock treatment. The genes on chromosome 3 were induced rapidly at 32 and 41 °C, whereas those on chromosome 1 were induced slowly by similar conditions (Guan et al., 2004).

Seven of these genes, except *Os*HSP16.9D and *Os*HSP17.9B, were induced by arsenite (As), but only genes on chromosome 3 were strongly induced by azetidine-2-carboxylic acid (Aze, a proline analog) and cadmium (Cd). A similar expression profile of all sHSP-CI genes at a lower level was suggested by ethanol, $H_2O_2$ and $CuCl_2$ treatments (Guan et al., 2004). Transient expression assays of the promoter activity by linking to GUS reporter gene also supported the *in vivo* selective expression of the sHSP-CI genes by Aze treatment. This indicates that the differential induction of rice sHSP-CI genes is most likely regulated at the transcriptional level. Only *Os*HSP16.9A abundantly accumulated in matured dry seed, suggesting additional prominent roles played by this HSP in development (Guan et al., 2003, 2004).

Chang et al. (2007), for the first time, reported a full-length cDNA clone for a class II sHSP, isolated from rice-etiolated seedlings heat shocked at 41 °C for 2 h. The coding sequence consists of 501 bp, and the clone encodes a protein of 18.0 kDa. The obtained full-length cDNA clone, designated *Os*HSP18.0-CII, is almost identical to a putative class II sHSP gene located on rice chromosome 1 and another putative class II sHSP rice gene. *Os*HSP18.0-CII was provoked by mechanical injury and salicylic acid treatment, which is not common in this class of sHSP genes. Siddique et al. (2003) reported a new class of plant sHSPs with dominant nuclear localization (HSP17-CIII). The corresponding proteins in tomato, *Arabidopsis*, and rice are encoded by unique genes containing a short intron in the beta4-encoding region of the α-crystallin domain (ACD). The strong nuclear localization results from a cluster of basic amino acid residues in the loop between beta5 and beta6 of the ACD. Proteins of the sHSP classes CI, CII, and CIII interact with each other, thereby influencing oligomerization state and intracellular localization. Oxidative stress as well as $Cu^{2+}$, induces the expression of the sHSPs α-crystallins. Other divalent cations such as $Zn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$ do not affect $Cu^{2+}$ binding, indicating selectivity of the $Cu^{2+}$-binding site(s) in α-crystallins. $Cu^{2+}$ binding induces structural changes and increase in the hydrodynamic radii of α-crystallins. The $Cu^{2+}$-sequestering (redox-silencing) property of α-crystallins confers cytoprotection and helps in understanding their protective role in neurodegenerative diseases and cataract (Ahmad et al., 2008).

It has been reported that sHSPs located in mitochondria and chloroplasts protect respiratory electron transport in mitochondria and photosystem II (PSII) electron transport in chloroplast (Heckathorn et al., 1999; Sun et al., 2002). Some sHSPs have been demonstrated to act as molecular chaperone *in vitro* and *in vivo*. It was reported that, 18.1 kDa sHSPs has been shown to prevent protein aggregation. Maintainance of the refolding is done by other chaperons. There is no evidence that sHSPs are required for normal cellular functions. They are typically found in heat stressed plants (Sun et al., 2002).

sHSPs are also produced in other environmental stresses and some developmental stages (embryogenesis, germination, pollen growth and fruit maturation). The induction of sHSPs gene expression and accumulation of sHSPs in environmental stress shows the importance of these proteins in response to stress (Ledesma and Kawabata, 2004; Dell' Aquila, 2000). After the heat stress

has been released, the sHSPs are quite stable with half-lives of 30-50 hours, suggesting that sHSPs may be important for recovery as well. sHSPs genes are expressed under control of the heat shock transcription factor (HSF). It has been believed that sHSPs play role in translational control on heat stress (Sun et al., 2002).

**Ubiquitin (HSP8.5 group):** Ubiquitin is a highly conserved, low molecular weight protein with 75-76 amino acid residues. It is present in every eukaryotic cell. It could be found either free or bound to various proteins by their terminal glycine amino acid (Lindquist and Craig, 1988). Heat stress induces synthesis of ubiquitin and thus, may have a vital function for stress tolerance and recovery. Plants use a repertoire of methods to control the level and activity of their constituent proteins (Nover et al., 1989).

## PROPOSED FUNCTIONS OF sHSPs

Production of high levels of heat shock proteins can also be triggered by exposure to different kinds of environmental stress conditions such as infection, inflammation, exercise, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation. Consequently, the heat shock proteins are also referred to as stress proteins and their regulation is sometimes described more generally as part of the stress response (Santoro, 2000).

### sHSPs as molecular chaperones

Small heat shock proteins function as intra-cellular chaperones for other proteins. They play an important role in protein-protein interactions such as folding. They assist in the establishment of proper protein conformation and prevent unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell (Walter and Buchner, 2002; Borges and Ramos, 2005). HSPs recognize and bind to other proteins when these other proteins are in non-native conformations. The non-native conformations of these proteins could be due to protein-denaturing stress or due to immature peptides folded, assembled, or localized to an appropriate cellular compartment. In the presence of ATP at normal physiological concentrations, sHSPs change their confirmation and releases denatured protein, allowing other molecular chaperones such as HSP70 to renature the protein and renew its biological activity. In the absence of ATP, sHSPs such as α-crystallin are more efficient than HSP70 in preventing stress-induced protein aggregation (Wang and Spector, 2001). *In vitro*, sHSPs selectively bind and stabilize proteins and prevent their aggregation at elevated temperatures in an ATP-independent way and protect enzymes against heat-induced inactivation (Ganea, 2001).

Typically, HSPs function as oligomers, if not as complexes of several different chaperones, co-chaperones or nucleotide exchange factors. Interaction with chaperones is responsible for (a) maintaining HSPs' partner proteins in a folding-competent, folded, or unfolded state; (b) organellar localization, import, and/or export; (c) minimizing the aggregation of non-native proteins; and (d) targeting non-native or aggregated proteins for degradation and removal from the cell (Feder and Hofmann, 1999). It was clearly revealed that the insoluble sHSP/substrate complex is formed when sHSP is overloaded with non-native substrates, which is the very case under *in vivo* conditions. The proposal that sHSPs function to prevent protein aggregation seems misleading. sHSPs appear to promote the elimination of protein aggregates by incorporating into the insoluble protein complex (Jiao et al., 2005). α-crystallin specifically protects enzymes against inactivation by different posttranslational modifycations such as glycation, carbamylation and aldehyde binding, and also reactivates GuHCl-denatured enzymes (Ganea, 2001).

Atungulu et al. (2006) reported that sHSP21 and sHSP23 of the leaf beetle, *Gastrophysa atrocyanea* display a double chaperone function *in vitro* critical for the survival of the leaf beetles against higher temperatures. sHSPs 21 and 23 that are constitutively expressed in the absence of stress during development in many organisms not only enhanced thermoresistance but can also play a role in developmental, tissue, and cell-specific induction and expression. The RNAi suppression of genes in adults resulted in decreased thermoresistance and show strong correlation between *in vitro* chaperone functions and *in vivo* thermotolerance analysis.

### sHSP in vascular relaxation

Heat shock proteins appear to serve a significant cardiovascular role. HSP90, HSP84, HSP70, HSP27, HSP20, and alpha beta crystalline have all been reported as having roles in the cardiovasculature (Benjamin and McMillan, 1998). HSP90 binds both endothelial nitric oxide synthase and soluble guanylate cyclase, which in turn are involved in vascular relaxation (Antonova et al., 2007). HSP20 phosphorylation correlates well with smooth muscle relaxation and is one significant phosphoprotein involved in the process (McLemore et al., 2005). HSP20 appears significant in development of the smooth muscle phenotype during development. HSP20 also serves a significant role in preventing platelet aggregation, cardiac myocyte function, prevention of apoptosis after ischemic injury, skeletal muscle function and muscle insulin response (Fan et al., 2005). HSP27 is a major phosphoprotein during muscle contraction. HSP27 functions in smooth muscle migration and appears to

serve an integral role (Salinthone et al., 2008).

In skeletal muscle, members of the a-crystallin domain-containing family of small heat shock proteins are believed to form a cohort of essential stress proteins. aB-crystallin (aBC/HSPB5) and the cardiovascular heat shock protein (cvHSP/HSPB7) are both implicated in the molecular response to fiber transformation and muscle wasting. The age-related loss of skeletal muscle mass and strength, now generally referred to as sarcopenia, is one of the most striking features of the senescent organism. The results were confirmed by immuno-fluorescence microscopy and immunoblot analysis, which showed a dramatic age-induced increase in these sHSPs. Other major stress proteins, showed that they were not affected or less drastically changed in their expression in aged muscle. Increase in muscle-specific sHSPs constitutes an essential cellular response to fiber aging and might therefore be a novel therapeutic option to treat sarcopenia of old age (Doran et al., 2007). Mounier and Arrigo (2002) reported that microfilament of the cytoskeleton and oligomers of the sHSPs exist in 2 reversible forms (a spherical and hollow form). Direct actin-sHSP interaction occurs to *in vitro* inhibition of actin polymerization, and *in vivo* regulation of actin filament dynamics. As both proteins share common structural motives, this interaction also protects the actin cyto-skeleton in which microfilaments would be coated by small oligomers of phosphorylated sHSPs when present as a nonphosphorylated monomer. It could also help in stabilizing and protecting the microfilament when organized in small, phosphorylated oligomers. In another report Ramakrishna et al. (2003), have characterized a novel sHSP gene, viscosity 1 (vis1) from tomato (*Lycopersicon esculentum*) and provided evidence that it plays a role in pectin depolymerization and juice viscosity in ripening fruits. Expression of vis1 is negatively asso-ciated with juice viscosity in diverse tomato genotypes. Vis1 is regulated by fruit ripening and high temperature and exhibits a typical HSP chaperone function when expressed in bacterial cells.

## sHSP and electron transport chain

Chloroplast sHSPs are expressed in leaves in response to a number of stresses such as drought, high light, UV-A, and oxidative stress. Chloroplast sHSPs protects photochemical system II (PSII) *in vitro* during photo inhibitory high light and oxidative stress (Downs et al., 1999b). *In vitro* experiments indicated that, chloroplast small heat-shock proteins (sHSPs) could associate with thylakoids and protect PSII during heat and other stresses, possibly by stabilizing the $O_2$ evolving complex (OEC) (Downs et al., 1999a). However, *in vivo* evidences show that PSII function decreased at high temperatures in the tolerant genotype. Differences in PSII thermo-tolerance *in vivo* were associated with increased thermo-tolerance of the OEC proteins and $O_2$ evolving functions of PSII, and not with other PSII proteins (Heckathorn et al., 2002). *In vitro* chloroplast sHSPs also protect photosynthetic electron transport from oxidative damage resulting from exogenous $H_2O_2$ (Downs et al., 1999c). PSII was the most thermosensitive component of photo-synthetic electron transport, and no differences between genotypes in the thermotolerance of other electron transport components were observed. These results indicated that, *in vivo* chloroplast sHSPs can protect $O_2$ evolution and the OEC proteins of PSII during heat stress (Heckathorn et al., 2002).

Mitochondrial sHSPs exhibits high similarity to chloroplast sHSPs in the C-terminal region but differs in the N-terminal region of the proteins (Waters et al., 1996). Mitochondrial sHSPs containing a partially con-served "Met-rich" domain may function as anti-oxidants, protect electron transport by a mechanism that is similar to that of the chloroplast sHSPs (Hamilton and Scott, 2001). During Sodium (Na) stress, mitochondrial sHSPs protect complex I electron transport because complex I is protected only by anti-oxidants, but not Complex II that is protected only by osmoprotectants. This suggests that mitochondrial sHSPs protect Complex I through an anti-oxidant mechanism (Downs et al., 1999b).

## sHSP and development

In animals, sHSP genes are developmentally regulated under both normal and environmental stress conditions. For example, in *Xenopus*, the sHSP gene family is repressed and not heat-inducible until the late neurula and early tailbud stage. Developmental regulation of these sHSP genes is controlled, in part, at the level of chromatin structure while other HSPs are inducible at the onset of zygotic genome activation at the mid blastula stage. During environmental stress, amphibian multimeric sHSPs bind to denatured target protein, inhibiting their aggregation and maintaining them in a folding-competent state until reactivated by other cellular chaperones (Heikkila, 2004).

In plants, sHSPs are developmentally induced during microspore embryogenesis independent of environmental stress. This occurs at low levels in the early unicellular stage (Waters et al., 1996) but increases by four to seven folds in matured pollen (Volkov et al., 2005). Cytosolic class I and II sHSP are developmentally expressed in maturing embryo of pea, wheat, sunflower, alfalfa, *Arabi-dopsis*, tobacco, maize and tomato in a stage-specific fashion. This suggests that, certain sHSP genes may play specific roles in early, others during later stages of pollen development (Waters et al., 1996). Heat stress, cold, and starvation, which induce microspore embryo-genesis, can modify sHSPs mRNA levels. However, only heat stress enhances the expression of sHSP in micro-spores but there is no correlation for the expression of

specific sHSP with the potential for microspore embryogenesis (Volkov et al., 2005).

In sunflower Ha HSP17.7 G4 mRNAs, accumulated during zygotic embryogenesis at 25°C and Ha HSP18.6 G2 mRNAs accumulated only in response to heat-shock. In vegetative tissues, these mRNAs accumulated in response to heat shock (42°C), abscisic acid (ABA), or mild water stress treatments. Developmental induction of Ha HSP17.7 G4 during zygotic embryogenesis was faithfully reproduced in the transgenic plants (Coca et al., 1996).

Puigderrajols et al. (2002) reported a transient accumulation of class I sHSPs during cork oak somatic embryo maturation and germination. This was localized to early differentiating, excluding the highly dividing regions of the root and shoot apical meristems. The amount of sHSPs increased at all stages of embryo development in response to exogenous stress. At mid maturation stage in pea embryos, class I and II sHSPs appear during the biosynthesis of storage proteins and increase during seed dehydration. At seed germination after emergence of the radical, sHSPs can be detected after 2 to 3 days (De Rocher and Vierling, 1994). In sunflower seeds, class I sHSPs is expressed during late seed maturation (Coca et al., 1994) and in *Arabidopsis* seeds, it accumulates at mid maturation and decline during germination (Wehmeyer et al., 1996).

## STRUCTURAL DYNAMICS OF sHSPs

sHSPs are organized into large, sphere-like structures commonly consisting of 12 or 24 subunits. Electron microscopy showed that HSP20.2 forms two distinct types of octahedral oligomers of slightly different sizes, indicating certain structural flexibility of the oligomeric assembly. Under conditions of heat stress, the distribution of the structurally different HSP20.2 assemblies changed, and this change was correlated with an increased chaperone activity. In analogy to HSP20.2, HSP 16.5 oligomers displayed structural dynamics and exhibited increased chaperone activity under conditions of heat stress (Haslbeck et al., 2008).

sHSPs share an evolutionarily conserved sequence of 80-100 amino acids, located in the C-terminal region, and called α-crystallin domain or heat shock domain contributing to subunits interactions (Plesofsky-Vig et al., 1992). The heat shock domain can be further subdivided into two regions; consensus region I and II separated by a hydrophilic region of variable length (Lindquist and Craig, 1988). The N-terminal consensus region I, preceding the α-crystallin domain, is variable in length and amino acid sequence. It consists of a conserved Pro-$X_{(14)}$-Gly-Val-Leu sequence, contributing to structural diversity between different sHSPs and having a role in multimerization (Lindquist and Craig, 1988; Vierling, 1991). The α-Crystallin domain is followed by C-terminal consensus region II. It consist of Motif Pro-$X_{(14)}$-X/Val/Leu/Ile-Val/Leu/Ile (Vierling, 1991), a polar structure that is involved in protein solubility and which share no sequence homology (Ganea, 2001).

All plant's sHSPs spontaneously form 200-300 KDa homooligomers *in vivo* and *in vitro*. Deletion of 15 amino acid residues of the N-terminal domain caused a dramatic reduction of the oligomer size indicating that this N-terminal region is involved in the oligomerization of sHSPs (Lee et al., 1995). Deletion of C-terminal region only reduces solubility of sHSPs oligomers but did not affect the oligomerization (Leroux et al., 1997). Basha et al. (2006) reported the role of the conserved C-terminal, a-crystallin domain, versus the variable N-terminal arm in substrate interactions by comparing two closely related dodecameric plant sHSPs, Hsp18.1 and Hsp16.9, and four chimeras of these two sHSPs, in which all or part of the N-terminal arm was switched. Different substrates have varying affinities for different domains of the sHSP. As for luciferase and citrate synthase, the efficiency of substrate protection is determined by the identity of the N-terminal arm in the chimeric proteins. In contrast, for malate dehydrogenase (MDH), efficient protection clearly required interactions with the a-crystallin domain in addition to the N-terminal arm. sHSP-substrate complexes with varying stability and composition can protect substrate equally. Substrate protection is not correlated with sHSP oligomeric stability for all substrates. Protection of MDH by the dimeric chimera composed of the HSP16.9 N-terminal arm and HSP18.1 a-crystallin domain supports the model that a dimeric form of the sHSP can bind and protect substrate.

## sHSPs AND DISEASES

sHSPs can prevent cataract in the mammalian lens and have protective effect against ischemic and reperfusion injury due to heart attack and stroke. During ischemia, proteins that are not in their final folding state binds to the large oligomeric small heat shock protein complexes. After the ischemia is resolved, HSP70 shuttled to a productive refolding pathway resulting in proteins in their final folding state, which can assume their normal activity in cells recovered from ischemic injury (Dillmann, 1999). On the other hand, mutated sHSPs are implicated in diseases such as desmin-related myopathy and they have an uncertain relationship to neurological disorders including Parkinson's and Alzheimer's disease (Sun and MacRae, 2005b).

Parcellier et al. (2005) has proposed mechanisms for the cytoprotective functions of small heat shock proteins. sHSPs are powerful molecular chaperones whose main function is to prevent the aggregation of nascent and stress-accumulated misfolded proteins and they interact directly with various components of the tightly regulated programmed cell death machinery, upstream and downstream of the mitochondrial events. sHSPs appear to play

a role in the proteasome-mediated degradation of selected proteins. Both HSP27 and aB-crystallin were also proposed to participate in the development of neurodegenerative diseases and malignant tumors in which their overexpression could induce drug resistance.

sHSPs were also overexpressed in various types of cancer, being associated with tumor cell proliferation, differentiation and apoptosis (Ciocca and Calderwood, 2005). However, the exact molecular mechanisms through which HSPs become overexpressed in cancer remain to be clarified (Giaginis et al., 2009). Ageing, cancer progression, vascular damage, diabetes, kidney and neuron degeneration, though unrelated in their etiology and clinical manifestation, represent states of increased oxidative stress, which in turn, promotes amorphous aggregation of target proteins, increased genomic instability and high rates of cellular death. Clusterin/ apolipoprotein J (CLU) has small heat shock proteins-like chaperone activity and its involvement in cell death regulations, which are both directly correlated to the main features of oxidant injury. The presence of both a heat shock transcription factor-1 and an activator protein-1 element in the CLU gene promoter indicate that CLU gene can be an extremely sensitive biosensor to reactive oxygen species (Trougakos and Gonos, 2006).

During carcinogenesis, oncoproteins such as mutated p53 and conformationally altered proteins may appear which may elicit an HSP response (Tang et al., 2005). HSP-27, -60, -90 proteins were abundantly expressed in gastric adenocarcinoma and associated with tumor size, patients' sex and associated with longer overall survival time (Giaginis et al., 2009).

## LOCALIZATION OF sHSPs

sHSPs are ubiquitous intracellular proteins and potently prevent the amorphous aggregation and precipitation of target proteins under stress conditions such as elevated temperature, reduction and oxidation without the need for hydrolysis of ATP (Carver et al., 2003). In the leaves of tomato (*Lycopersicon esculentum* Mill.) mitochondrial small heat-shock protein (MT-sHSP) genes quickly respond to heat stress but not or weakly expressed at a normal growth temperature. In the flowers mitochondrial and endoplasmic reticulum (ER), localized small heat-shock proteins were accumulated in the ovule, but not in the pollen (Sanmiya et al., 2005). sHSPs are also found in storage organs like tendrils of *Aristolochia,* twigs of *Acer and Sambucus,* and bulb of *Crocus, Allium, Amaryllus* and *Hyacinthus* (Prandl et al., 1995).

Western blot analysis showed no significant amount of the 17.0 kDa sHSP in non-stressed vegetative tissues, and upon heat shock it accumulates to levels comparable to those constitutively found in embryo tissues from mature seeds (Schubert et al., 2002). Verschuure et al. (2002) reported that sHSPs (aB-crystallin and HSP25) colocalize with F-actin upon proteasomal inhibition and translocate from the detergent-soluble cytosolic fraction to the detergent-insoluble nuclear/cytoskeletal fraction. Other sHSPs (aA-crystallin, HSP20, HSPB2 and HSPB3) showed similar translocation to the actin cytoskeleton. Dafny-Yelin et al. (2008) reported that the rose (*Rosa hybrida*) cytoplasmic 17.5-kDa Class I small HSP increases dramatically during flower development, and accumulates in closed bud petals and leaves only in response to heat stress. A mRNA for a putative ortholog of this protein is also found in petals, but not from leaves, of *Arabidopsis thaliana* plants grown under optimal conditions. It accumulates in leaves in response to heat stress. In another report, Doerwald et al. (2004) reported head-to-head gene pair encoding two small heat shock proteins, aB-crystallin and HspB2. This is closely linked in all major mammalian clades, suggesting that this close linkage is of selective advantage. aB-crystallin is abundantly expressed in lens and muscle and in response to a heat shock, while HspB2 is abundant only in muscle and not up-regulated by a heat shock.

## MOLECULAR EVOLUTION OF sHSPs

The small heat-shock proteins have a tremendous diversification in plants in both sequence and cellular localization. They are encoded by at least five gene families. sHSPs are found in all three domains of life (Bacteria, Archaea, and Eukarya) and particularly diverse within land plants and the evolutionary origin of the land plant sHSP families is still an open question (Waters and Rioflorido, 2007). The different evolutionary histories of the large and small HSPs suggest that, even if both types of HSPs are molecular chaperones, the specific functions within the cell and the selective constraints on these groups of proteins are very different (Waters, 1995). Unlike the large HSPs (represented by Hsp60 and Hsp70), sHSPs are highly divergent in both primary sequences and oligomeric status, with their evolutionary relationships being unresolved (Fu et al., 2006).

Higher plants contain over 20 different small heat shock proteins whereas only a single small heat shock protein is found in fungi and many animals. The phylogenetic relationships reveal that sHSPs are evolved due to gene duplication, sequence divergence and gene conversion at different rates (Waters, 1995). Phylogenetic analysis of a representative 51 sHSPs (covering the six subfamilies: bacterial class A, bacterial class B, archae, fungi, plant, and animal) reveals a close relationship between bacterial class A and animal sHSPs which form an outgroup. Oligomers from bacterial class A and animal sHSPs appear to exhibit polydispersity, while the others exhibit monodispersity suggesting a potential evolutionary origin (Fu et al., 2006).

Ten known sHSPs have being reported in mammalians. This group was formally named HspB1-B10 out

of which only three of them (Hsp27/B1, aA-crystallin/B4, aB-crystallin/B5) have been reported in nonmammalian vertebrates. However, an apparent paralog, Hsp30/B11, is found in frogs and teleost fish. Orthologs of seven mammalian sHSPs is now found in other vertebrate classes. Two novel sHSPs, named HspB11 and HspB12, were recognized in birds, and four novel sHSPs, named HspB12-B15, in teleost fish (Franck et al., 2004). The 15 recognized paralogous vertebrate sHSPs reflect the period of extensive gene duplications early in vertebrate evolution. It is inferred that at least 13 intron insertions have occurred during the evolution of chordate sHSP genes, while a single ancient intron is maintained in some lineages, in line with the general trend of massive intron gain before or during early vertebrate radiation. The occurrence of several head-to-head located pairs of chordate sHSP genes is quite interesting (Franck et al., 2004).

Waters and Vierling (1999) reported evolutionary history of the chloroplast (CP) localized small heat shock proteins (sHSPs) in plants, only identified from angiosperms. Phylogenetic analysis of the CP sHSPs with other plant CP sHSPs and eukaryotic, archaeal, and bacterial sHSPs showed that the CP sHSPs are not closely related to the cyanobacterial sHSPs. Thus, they most likely evolved via gene duplication from a nuclear-encoding cytosolic sHSP and not via gene transfer from the CP endosymbiont. Previous sequence analysis had shown that all angiosperm CP sHSPs possess a methionine-rich region in the N-terminal domain. The primary sequence of this region is not highly conserved in the *F. hygrometrica* CP sHSPs. This lack of sequence conservation indicates that sometime in land plant evolution, after the divergence of mosses from the common ancestor of angiosperms but before the monocot-dicot divergence, there was a change in the selective constraints acting on the CP sHSPs. This tremendous diversification of small heat-shock proteins in plants may reflect adaptations to stresses unique to plants (Vierling, 1991).

Diversity of algal sHSPs is not correlated with adaptation to extreme conditions. All of the algal sHSPs identified are members of this large and important superfamily but not the diverse land plant sHSP families. The evolutionary relationships among the algal sHSPs and homologues from bacteria and other eukaryotes are consistent with the hypothesis that the land plant chloroplast and mitochondrion sHSPs did not originate from the endosymbionts of the chloroplast and mitochondria (Waters and Riofloride, 2007).

## sHSP GENES EXPRESSION

*Oryza sativa* heat shock protein (OsHSP) genes exhibited diverse expression in different organs and tissue. Specific expression patterns were different from gene to gene even when they belong to the same family. Some plant sHSPs have been shown to be a part of normal development during pollen development, seed maturation, seed imbibition and germination. *Oryza sativa* 3 sHSPs, (OsHSP17.0, OsHSP26.7 and OsHSP24.1) were predominantly expressed in spikes and/or imbibed seed embryos. They would play certain roles in pollen development and seed germination. OsHSP70 and OsHSP71.1 were highly expressed during seed imbibition (Zou et al., 2009). Over expression of *Oryza sativa* sHSP 17.7 (Murakami et al., 2004) and sHSP 18.0 (Chang et al., 2005), also increases the degree of resistance to UV damage in transgenic rice and *Escherichia coli* respectively. sHSPs of Chestnut (*Castanea sativa*) CsHSP 17.5 belonging to cystolic class I, enhance the cell survival against both thermal extremes (50 and 4℃) (Soto et al., 1999).

The molecular chaperone activity of this gene may play essential roles during seed germination. Usually, reproductive organs are much more sensitive to heat stress than vegetative organs. Heat stress that can result in severe rice yield losses happened during the pollen development to embryo developmental stages (Zou et al., 2009). The correlation between sHSP-CI accumulation, expansion of rose petal cells, impairment of acquired thermotolerance, and defects in early embryogenesis of the double mutants (hsp17.4/hsp17.6A), all suggest that sHSP-CI proteins play a role in protecting cell proteins at various developmental stages. While in hypocotyl elongation they have a non-redundant function in acquired thermo-tolerance, in early embryogenesis they have a redundant function (Dafny-Yelin et al., 2008).

The heat-induced expression levels of Hsp17.6 and Hsp18.2 which increases intracellular accumulation of $H_2O_2$ in *Arabidopsis* cell culture were significantly reduced in the presence of ascorbate or 2,6 dichlorophenolindophenol (DPI), indicating that $H_2O_2$ is an essential component in the heat stress signaling pathway (Volkov et al., 2006). Two related small HSPs, HSP25 and aB crystallin were accumulated in the eye lens and many normal adult tissues like heart, stomach, colon, lung, and bladder of mice even in the absence of heat stress. The stress-independent expression pattern of these two small HSPs is distinct. This amount is increased in fibroblasts in response to heat stress. The expression of HSP25 and aB crystallin in normal tissues suggests an essential, but distinct function of these two related proteins under standard physiological conditions (Klemenz et al., 1993).

Wehmeyer and Vierling (2000) reported on sHSP expression in wild-type and seed maturation mutants of *Arabidopsis* by western analysis using an HSP17.4 promoter-driven beta-glucuronidase (GUS) reporter gene in transgenic plants. Examination of HSP17.4 expression in seeds of the transcriptional activator mutants abi3-6, fus3-3 and lec1-2, showed that protein and HSP17.4: GUS activity were highly reduced in fus3-3 and lec1-2

and undetectable in abi3-6 during distinct developmental and stress regulation. Quantification of sHSP in these mutant seeds showed that all had < 2% of HSP17.4 levels. This reduction suggested that sHSPs have a general protective role throughout the seed (Wehmeyer and Vierling, 2000; Wolkers et al., 1998).

Doerwald et al., (2003) reported the overexpression of aB-crystallin or HSP27, cap-dependent initiation of translation was protected but no effect was seen on cap-independent initiation of translation while HSP70 was over-expressed. However, both cap-dependent and independent translation were protected which indicates a difference in the mechanism of protection mediated by small or large heat shock proteins.

## APPLICATIONS OF sHSPs

Han et al. (2005) reported that sHSPs from *Escherichia coli* and *Saccharomyces cerevisiae* are able to protect proteins *in vitro* from proteolytic degradation in two-dimensional electrophoresis (2-DE). Addition of sHSPs during 2-DE of whole cell extracts of *Arabidopsis tha--liana*, *E. coli*, *Mannheimia succiniciproducens*, and human kidney cells allowed detection of up to 50% more protein spots than those obtainable with currently available protease inhibitors. Therefore, the use of sHSPs during 2-DE significantly improves proteome profiling by generally enabling the detection of many more protein spots that could not be seen previously. The amphitropic sHSPs associate with membranes although they do not contain transmembrane domains or signal sequences. Recent studies indicate that small heat shock proteins play an important role in membrane quality control and thereby potentially contribute to the maintenance of membrane integrity especially under stress conditions (Nakamoto and Vigh, 2007).

## sHSP PROMOTERS

Eukaryotic promoters are extremely diverse and are difficult to characterize. They typically lie upstream of the gene and can have regulatory elements several kilobases away from the transcriptional start site (enhancers). In eukaryotes, the transcriptional complex can cause the DNA to bend back on itself, which allows for placement of regulatory sequences far from the actual site of transcription. Many eukaryotic promoters (between 10 and 20% of all genes), contain a TATA box sequence (TAT AAA), which in turn binds a TATA binding protein which assists in the formation of the RNA polymerase transcriptional complex (Smale and Kadonaga, 2003). The TATA box typically lies very close to the transcriptional start site, often within 50 bases.

The promoter of a sHSP gene from sunflower Ha HSP17.7 G4 was developmentally regulated during zygotic embryogenesis and responded to heat stress in vegetative tissues (Almoguera et al., 1998; Escobar et al., 2003). Mutations in the heat shock element (HSE) affected nucleotides crucial for human heat shock transcription factor 1 (HSF1) binding. This mutation abolished the heat shock response of Ha HSP17.7 G4 and produced expression changes that demonstrated dual regulation of this promoter during embryogenesis. Activation of the chimeric genes during early maturation stages does not require intact HSE. Expression at later desiccation stages was reduced by mutations in both the proximal (-57 to -89) and distal (-99 to -121) HSE. In contrast, two point mutations in the proximal HSE that did not severely affect gene expression during zygotic embryogenesis eliminated the heat shock response of the same chimeric gene in vegetative organs. Therefore, by site-directed mutagenesis, it was possible to separate the heat shock response of Ha HSP17.7 G4 from its developmental regulation (Almoguera et al., 1998).

Carranco et al. (1999) reported the existence of other cis-acting regulatory elements different from the HSE in upstream promoter region of sunflower Ha HSP17.6 G1which might have positive or negative quantitative effects on seed-specific expression of the Ha HSP17.6 G1. In sunflower Ha HSP17.7 G4 5'-distal sequences (between -1132 and -395) were required to confer a preferential spatial expression of GUS activity in the cotyledons. More proximal sequences (from -83 to +163) conferred to the chimeric genes most of the developmental regulation, and the responses to ABA and heat shock characteristic of the Ha HSP 17.7 G4 promoter (Coca et al., 1996).

In rice, 1.6-kb DNA fragment encoding a 16.9-kDa class-I sHSP was composed of 365-bp tandem direct repeats (DRs) and 441-bp near perfect long terminal inverted repeats (LTIRs). The DRs contain 123-bp regions with 99% nucleotide identity to the 5' coding region of the *Os*hsp16.9B gene. This fragment in proximity 5'-upstream of the *Os*HSP16.9B gene that was mapped on chromosome 1 with other two class-I sHSP genes, *Os*HSP16.9A and *Os*SHP16.9 C. Comparative analysis of the nucleotide sequences of class-I sHSP genes clustered on chromosome 1 showed that only the promoter region of *Os*HSP16.9B was different (Guan et al., 2003).

The intergenic distance between the genes for two small heat shock proteins, aB-crystallin and HSPB2 proteins in mammals, ranges from 645 bp (platypus) to 1069 bp (opossum), with an average of about 900 bp. In chicken, the distance was the same as in duck (1.6 kb). Phylogenetic footprinting and sequence alignment identified a number of conserved sequence elements close to the HSP B2 promoter and two farther upstream. All known regulatory elements of the mouse aB-crystallin promoter were conserved, except in platypus and birds. Deletion of the middle of the intergenic region including the upstream enhancer, affected the activity of both the rat aB-crystallin and the HSP B2 promoters. This suggests

the sharing of the enhancer region by the two promoters (Doerwald et al., 2004).

Unlike angiosperm sHSP genes, those from *Picea abies* contain two introns, located in the 5' untranslated and coding region, respectively. Homologous introns exist in sHSP genes from *Picea glauca*, and *Funaria hygrometrica*, suggesting the presence of introns as a retained primitive condition of plant sHSP gene evolution (Schubert et al., 2002). Heat shock genes expression is mainly attributed to the activation of heat shock factors (HSF), which as trimers recognize the highly conserved HSE, which has been defined as adjacent and inverse repeats of the motif 5'-nGAAn-3' such as 5'-nGA AnnTTCnnGAAn-3' (Schoffl et al., 1998). Formation of heat shock promoter element (HSE) protein-binding complex in heat-stressed or $H_2O_2$ treated cells and its inability to form this complex after ascorbate treatment suggests that, oxidative stress affects gene expression via HSF activation. Thus, $H_2O_2$ is involved in HSF activation during the early phase of heat stress. The heat stress induction of a high mobility HSE-binding complex, characteristic for later phase of heat shock response was blocked by ascorbate. $H_2O_2$ was unable to induce this complex suggesting that $H_2O_2$ is involved only in the early stages of HSF activation (Volkov et al., 2006).

Transcriptional regulation of small heat-shock proteins (sHSPs) depends on the particular combinations of heat stress cis-elements heat-shock elements (HSEs). The Ha HSP 17.7G4 (G4) promoter was active in giant cells and its HSE arrangements were crucial for this activation. The HaHSP17.6G1 (G1) promoter, not induced by heat shock, was silent in giant cells, while HaHSP 18.6G2 (G2), which responds to heat shock, was specifically induced in giant cells. The responses of the different promoters correlated with distinct HSE configurations, which might have implications on differential trans-activation. Furthermore, the shortest giant cell and heat-shock-inducible sHSP promoter version analyzed in tobacco (-83pb HaHSP 17.7G4) fully maintained its expression profile in *Arabidopsis*. Cyst nematodes did not induce the Hahsp17.7G4 promoter, revealing additional specificity in the nematode response (Barcala et al., 2008). The birds lack lens-specific region 1 (LSR1) and the heat shock elements (HSEs) while primordial mammalian aB-crystallin promoter had two LSRs and two HSEs (Doerwald et al., 2004).

Koide et al. (2006) reported on the transcription start sites of six heat shock-inducible genes and analyzed their promoter regions, which contain a putative consensus for $\sigma^{32}$ promoters in *Xylella fastidiosa* and up-regulation of virulence-associated genes such as *vapD* and genes for hemagglutinins, hemolysin, and xylan-degrading enzymes, which may indicate the importance of heat stress to bacterial pathogenesis.

Swamynathan and Piatigorsky (2002) reported the contribution of the intergenic enhancer to sHSP/aB-crystallin and Mkbp/HSPB2 promoter activity using dual-reporter vectors in transient transfection and transgenic mouse experiments. Deletion of the enhancer reduced sHSP/aB-crystallin promoter activity by 30- and 93-fold and Mkbp/HSPB2 promoter activity by 6- and 10-fold in transiently transfected mouse lens a-TN4 and myoblast C2C12 cells, respectively. Inversion of the enhancer, reduced sHSP/aB-crystallin promoter activity by 17-fold, but did not affect Mkbp/sHSPB2 promoter activity in the transfected cells. The orientation dependence and preferential effect of the sHSP/aB-crystallin intergenic enhancer on the sHSP/aB-crystallin promoter provide an example of adaptive changes in gene regulation and functional diversification of duplicated genes during evolution.

## CONCLUSION AND FUTURE PROSPECTIVE

This review gives a comprehensive overview of the current knowledge of structural dynamics, localization, function, molecular evolution and gene expression of sHSPs. These are important proteins for living systems and current evidences show that under stress sHSPs act as intra-cellular chaperones and prevent unwanted protein aggregation and when stress is release, it renature the proteins and renew its biological activity. sHSPs show extraordinary significance in normal cells and tissues by its presence at key stages of development like microspore embryogenesis, pollen maturation, embryo germination and highly dividing, regions of the root and shoot apical meristems among others.

sHSPs family contributes to variable views of structure-function relationship, which could be overcome by advances in X-ray crystallography and crystallization. In the absence of precise 3D structural data, various spectroscopic and computer analysis may be misleading about the structure and function of sHSPs. In addition, some very important questions for the future prospective have been raised in this paper. This include the timing of the origin or evolution of sHSP genes family, occurrence and importance of genes mutation or duplication and their regulation under the control of different types of heat shock elements (HSE). Understanding the role of sHSPs in relation to heat stress is a more applied prospective of its importance as a potential indicator. A combination of genomics such as microarray, PCR, and proteomics such as 2D gel electrophoresis, X-ray crystallography will further elucidate the effect of stress on expression pattern at DNA, mRNA and protein level.

## ACKNOWLEDGEMENT

This research was partially supported by Higher Education Commission, Islamabad Pakistan, through grand number 1113 of National Research Program for Universities.

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