Heat Shock Proteins 10
Series Editors: Alexzander A.A. Asea · Stuart K. Calderwood

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Heat Shock Proteins and Plants

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Heat Shock Proteins

Volume 10

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Heat Shock Proteins: key mediators of Health and Disease. Heat shock proteins (HSP) are essential molecules conserved through cellular evolution required for cells to survive the stresses encountered in the environment and in the tissues of the developing and aging organism. These proteins play the essential roles in stress of preventing the initiation of programmed cell death and repairing damage to the proteome permitting resumption of normal metabolism. Loss of the HSP is lethal either in the short-term in cases of acute stress or in the long-term when exposure to stress is chronic. Cells appear to walk a fine line in terms of HSP expression. If expression falls below a certain level, cells become sensitive to oxidative damage that influences aging and protein aggregation disease. If HSP levels rise above the normal range, inflammatory and oncogenic changes occur. It is becoming clear that HSP are emerging as remarkably versatile mediators of health and disease. The aim of this series of volumes is to examine how HSP regulation and expression become altered in pathological states and how this may be remedied by pharmacological and other interventions.

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Preface

The sedentary nature of plants constantly exposes them to extreme climatic changes in various geographic regions. Their ability to overcome these adverse conditions, grow, and thrive is a result of evolutionary changes in various morphological and physiological mechanisms that enable plants to survive extremely stressful conditions.

The importance of plants in human survival cannot be overstated, not only in terms of an important source of food, but also for its critical therapeutic value. Since the dawn of time, the use of plants for therapeutic purpose has been recorded in all major cultures.

Heat-shock proteins (HSP) are stress proteins known to provide cytoprotection and play important roles in protein folding/unfolding and modulate cellular immune responses. HSP are found in all plant species and are associated to plant biotic stresses and are often referred to as stress defense proteins. In addition, HSP serve a critical role in the plant's response against key crop phytopathogens around the world.

Heat-Shock Proteins and Plants provides the most up-to-date and concise reviews and progress on the role of heat-shock proteins in plant biology, structure, and function and is subdivided into chapters focused on Small Plant HSP (Part I), Larger Plant HSP (Part II), and HSP for Therapeutic Gain (Part III). This book is written by eminent leaders and experts from around the world and is an important reference book and a must-read for undergraduate, postgraduate students, and researchers in the fields of agriculture, botany, crop research, plant genetics and biochemistry, biotechnology, drug development and pharmaceutical sciences.

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Contents

Part I
Small Plant Heat Shock Proteins (HSP)

Chapter 1
Small Heat Shock Proteins: Roles in Development, Desiccation Tolerance and Seed Longevity

Harmeet Kaur, Bhanu Prakash Petla, and Manoj Majee

Abstract Small heat shock proteins are one of the five classes of heat shock proteins, a family named after their expression in response to heat shock. Despite their name some members of this family have been shown to express during a gamut of non-stress conditions in a variety of plant species. Small HSPs have been known to accumulate during plant developmental stages like pollen development, seed maturation stages, early seed germination and also in storage organs. Interestingly, aging induced accumulation of small HSPs has also been observed in a few species. The spatial and temporal accumulation pattern of small HSPs also correlates well with other seed abundant proteins like late embryogenesis abundant (LEA) proteins. Regulation of these developmental stages responsive and non-stress induced small HSPs is also distinct from the heat stress regulated transcript induction in terms of involvement of some novel and exclusive transcription activators like ABI3 and HsfA9. Small HSPs are known to function as molecular chaperone and thus their role in plant development especially during seed development has been discussed in the light of their functional implication during these stages.

Keywords Pollen • Seed development • Chaperone • LEA protein • Glassy matrix

Abbreviations

ACD	alpha crystalline domain
ATP	adenosine triphosphate
CDT	controlled deterioration treatment
DAP	days after pollination
DPI	days post imbibition

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GUS β-glucuronidase
HSE heat shock element
HSF heat shock transcription factor
HSP heat shock proteins
LEA late embryogenesis abundant
ROS reactive oxygen species

1.1 Introduction

Small heat shock proteins (HSPs) are the major class of heat shock protein repertoire during heat stress in plants. This class typically contains a highly conserved ~90 amino acid long alpha crystalline domain (ACD) at the c-terminal end (Waters et al. 1996). This class comprises of heat shock proteins ranging between 15 and 42 kDa in monomeric molecular weight, although they exist as large oligomeric assemblies of 2–48 subunits (Vierling 1997; Waters et al. 1996; Basha et al. 2012). These proteins are encoded by six nuclear gene families in plants making them the most complex class of HSPs in plants. These families vary on the basis of sequence similarity among member genes and their respective intracellular localization. Two cytosol localized (CI and CII) and rest organelle localized (e.g. Endoplasmic reticulum, chloroplast, mitochondrial) small heat shock protein families have been identified in plants (Waters et al. 1996). Small HSPs act by binding to cellular proteins during stress conditions and prevent their aggregation or misfolding. Although they are not themselves involved in folding they assist by maintaining the target proteins in a conformation which can be readily refolded once the constraining factors cease to exist (Lee et al. 1995, 1997). Earlier, small HSPs were thought to express almost exclusively in vegetative tissues only under heat stress, but studies have demonstrated the role of these proteins in diverse stresses like cold, dehydration, salinity and oxidative stress (Waters et al. 1996; Wang et al. 2004, 2005). A plethora of studies both in animal and plant systems provide evidence for the physiological role of Heat shock proteins in general and small HSPs in particular during developmental processes (Waters et al. 1996).

Small HSPs are functional molecular chaperones and protect the substrate proteins against thermal aggregation or denaturation (Lee et al. 1995, 1997). These proteins are highly capable of binding substrate proteins with non-native conformations. This binding possibly involves hydrophobic interactions and does not require ATP thus making them ATP independent chaperones. Although they are not directly involved in refolding reactions and only facilitate refolding by ATP dependent chaperones (Ehrnsperger et al. 1997; Lee et al. 1997; Lee and Vierling 2000).

1.2 Small HSPs and Their Role in Development

Small heat shock proteins (sHSPs) are ubiquitously produced by all organisms including plants in response to increased temperature and certain other stresses to protect organisms from stress induced damage or to repair damage caused by stress

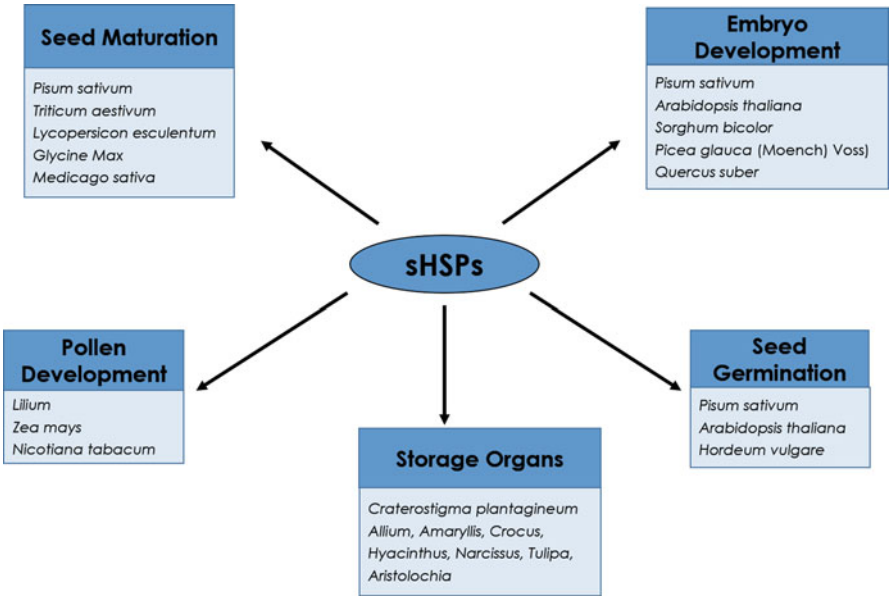

Fig. 1.1 Role of small HSPs during plant development processes

(Lindquist and Craig 1988; Vierling 1997). Recent studies have demonstrated the function of small HSPs during development in plants which range from pollen development, seed development and germination to their accumulation in storage organs as well (Fig. 1.1). These developmental processes related roles of small HSP family are discussed here in greater detail.

1.2.1 Role of Small HSPs in Pollen Development

Small HSPs have been shown to play important role during various developmental stages in the plant. Class II small HSPs have also been shown to be expressed in meiotic microsporocytes in lily (Bouchard 1990) and in developing maize anthers (Bouchard and Walden 1990; Dietrich et al. 1991). The transcript of one such gene *HSP18* was found to accumulate during meiosis phase of pollen development in lily and maize (Bouchard 1990; Bouchard and Walden 1990). This mRNA accumulation was observed in absence of high temperature and was independent of the heat stress mediated induction of small HSP mRNAs. The role of sHSPs in developmental stages was also observed as the higher expression of HSP18 in yeast (*Saccharomyces cerevisiae*) during late prophase of meiosis (Kurtz et al. 1986). Studies on plants, yeast and Drosophila meiotic stages linked accumulation of HSPs sheds light on the development related roles in meiotic cells (Winter and Sinibaldi 1991). Later studies done in tobacco by Zarsky et al. (1995) clearly demonstrated

the transcriptional activation of a small HSP gene, *NtHSP18P* in the course of initiation of embryogenesis in pollen. *NtHSP18P* mRNA was shown to be induced at normal non heat stress temperatures just before anthesis, which is marked by a dehydration phase during pollen development. A decade later Volkov et al. (2005) analysed the differential accumulation of small HSPs during pollen development and heat stress in tobacco. They identified eleven cytosolic small HSP coding cDNAs which were not expressed under normal temperatures but exclusively during pollen development. The protein products of these genes were also shown to be expressed throughout pollen developmental stages in a coordinated manner during early and late stages of pollen development.

1.2.2 Role of Small HSPs in Seed Development

Small HSP family of proteins are not only synthesised in response to stress conditions but also have a developmental response in organs like seeds. First reports of the presence of small HSP transcripts in seeds came from the work in *Pisum sativum* (DeRocher and Vierling 1994, 1995) where these transcripts were present in the embryonic axes and cotyledons of pea seeds and declined rapidly within a few hours of germination. Helm and Abernethy (1990) studied the HSP levels in wheat embryos and dry seed and described the presence of transcripts as well as proteins from several classes of HSPs in addition to the small HSPs. Vierling and Sun (1989) also identified few class I cytosolic mRNAs in pea seeds and similar observation was made in tomato fruits during ripening (Fray et al. 1990).

Cytoplasmic classes of small heat shock protein have been shown to accumulate at slightly elevated temperatures in pea and alfalfa indicating their role in heat stress adaptation (DeRocher et al. 1991; Hernandez and Vierling 1993). However, Hernandez and Vierling (1993) studied the accumulation of class I cytoplasmic small HSPs in various legumes viz. *P. sativum, G. max, V. unguiculata, P. acutifolius, M. sativa* and *A. constricta*, both under stress and in different plant organs. Their results showed the expression of small HSPs even in plants grown in field or green house under optimal growth conditions. They also showed that in addition to temperature stress, small HSPs normally express during certain developmental stages like in flower and seed development. Interestingly, these HSPs were absent in young pods but started accumulating at 29DAP and were also found in dry seeds and this expression is deemed important for their reproductive success.

1.2.3 Role of Small HSPs During Seed Germination

Studies demonstrate that developmentally induced small HSP (HSP17.6 and HSP17.9) mRNAs in sunflower seeds disappeared soon during imbibition but the proteins were interestingly present at elevated levels even 3 days post imbibition

(Coca et al. 1994). Although the major seed storage proteins degrade uptil 3DPI, the HSP17.6 and HSP17.9 protein products disappear afterwards. The persistence and organellar localization of these proteins hints at their involvement in seed reserve mobilization as discussed by Coca et al. (1994). In pea, small HSPs continue to exist in the cotyledons and axes for a number of days during imbibition and early germination before coming to a decline (DeRocher and Vierling 1994). Interestingly, barley HSP26 protein which is localized to plastid is found to accumulate in early germination stage which might have been translated from the HSP26 mRNA present in the early embryo (Kruse et al. 1993). Wheat chloroplastic HSP26 has also been shown to have high transcript during seed germination which was also confirmed by promoter: GUS construct expression. GUS expression driven by *TaHSP26* promoter was high during first 24 h after seed germination and declined later highlighting its role in early seed germination phase (Chauhan et al. 2012).

1.2.4 Role of Small HSPs in Storage Organs

Small HSPs have not only been shown to be involved in meiosis, microsporogenesis, seed development, seed germination and somatic embryogenesis, they have also been demonstrated to accumulate in vegetative organs not experiencing heat stress e.g. In desiccation tolerant plant *Craterostigma plantagineum*, polypeptides crossreactive to small HSP antibodies were detected in roots and lower shoot parts and similar observations were also made for mulberry where small HSP accumulates in response to seasonal cold acclimation in the endoplasmic reticulum enriched fractions of cortical parenchyma cells (Alamillo et al. 1995; Ukaji et al. 1999). Interestingly, small HSPs have been detected in bulbs and tubers of a variety of species like *Allium*, *Amaryllis, Crocus, Hyacinthus, Narcissus, Tulipa* and potatoes and also in twigs of *Acer* and *Sambucus* and tendrils of liana, *Aristolochia*. The expression in twigs and tendrils coincides with the dormant period in winter and fades out in spring. Potato tubers and *Aristolochia* tendrils were shown to have high proteins content which was located in the central vacuoles of storage parenchyma cells. A possible functional correlation was investigated between the accumulation of storage proteins and small HSPs and studies on transgenic tobacco plants demonstrated that in embryos of these plants which lack protein bodies and accumulation of storage proteins, small HSPs were also absent (Lubaretz and Niedin 2002). Interestingly, this association among the storage proteins and accumulation of small HSPs at the same stage also reflects in mature seeds where both these protein families are known to coexist and their expression has also found to be in sync (Almoguera and Jordano 1992; Coca et al. 1994) which is discussed in more detail later in this chapter. Thus, the existence of small HSPs in storage organs of perennial plants appears to have a correlation with the presence of other storage proteins (Lubaretz and Niedin 2002).

1.3 Molecular Regulation of Small HSP Accumulation During Development

Heat stress leads to a rapid increase in HSP gene transcription most of them being the small HSPs and the mRNA levels reach 20,000 copies in a cell (Schöffl and Key 1982). Heat shock elements (HSE) are the cis acting elements present in the 5′ upstream regions of the heat shock responsive genes and are known to confer heat shock induced expression of these genes. These HSE provide binding sites for heat shock transcription factors thus regulating the heat stress induced transcription activation (Goldenberg et al. 1988; Parker and Topol 1984; Wu 1984a, b). This heat stress responsive role of small HSP family proteins was complemented by the developmental stage specific expression of certain members of this family as transcripts for some small HSPs have been detected in wheat, sunflower and pea grown in the absence of heat stress (Helm and Abernethy 1990; Almoguera and Jordano 1992; Coca et al. 1994; DeRocher and Vierling 1994; Carranco et al. 1997).

Developmental and heat stress regulated expression of small HSPs might be overlapping but should be distinctly controlled as not all small HSP classes/members are developmentally expressed. In order to decipher these complex regulatory mechanisms, promoters and 5′ upstream regions were studied from small HSP genes expressed in response to both heat stress and development. Promoter studies with promoter: GUS reporter constructs made with two such genes from soybean and sunflower (*GmHSP17.3B* and *HaHSP17.7G4*) demonstrated their expression during zygotic embryogenesis (Prändl et al. 1995; Prändl and Schöffl 1996; Coca et al. 1996; Carranco et al. 1999). Subsequent studies on *HaHSP17.4G4* promoter transgenic lines revealed the involvement of two discrete regulatory mechanisms, an HSE/HSF dependent mechanism and another HSE independent mechanism suggested to be regulated by developmental stage specific novel transcription activators (Almoguera et al. 1998). Experiments with different mutations within the HSE core element did not affect the tissue specific expression pattern of the GUS reporter gene during early seed maturation however the binding between this mutated HSE and the HSF transcription factor was highly compromised. Thus the seed maturation stage responsive transcription by *HaHSP17.4G4* promoter was determined to be HSE independent (Almoguera et al. 1998). One such example for the developmental regulation of small HSP family genes in animals comes from larval development in Drosophila where the promoters of HSP23 and HSP27 genes are regulated by ecdysterone receptors. These ecdysterone receptors have DNA

binding domain and are activated by addition of ecdysterone hormone. This type of activation is independent of HS induced HSE mediated activation of HSPs (Luo et al. 1991). Similar evidence comes from the study of Arabidopsis seed mutants, *aba1* and five *abi* loci mutants, (Wehmeyer et al. 1996) in which *abi3* mutants had lower accumulation of small HSPs and the *abi3* null mutants were found to be desiccation intolerant. Although studies with other *abi* mutants showed the presence of wild type levels of small HSPs; but other desiccation intolerant Arabidopsis mutants *abi3-6, fus3-3, lec1-2* and line 24 did have severely reduced levels of HSP17.4 (major small HSP present in Arabidopsis seeds). Therefore it can be concluded that small HSPs may not be solely responsible for providing desiccation tolerance but are definitely necessary for seed desiccation tolerance (Wehmeyer et al. 1996; Wehmeyer and Vieling 2000). Apart from HSF, some other transcriptional activators are also speculated to be involved in seed developmental regulation of small HSPs.

In Arabidopsis, ABI3 has been implicated as one such activator of *HSP17.4* during seed development. Wehmeyer and Vierling (2000) showed the levels of *HSP17.4* to be untraceable in the *abi3-6* knockout mutant. Promoter: GUS constructs transformed in *abi3-6* mutant background also demonstrated a very faint GUS staining in seeds which reveals very low transcriptional activation in the absence of ABI3 activator. However, in other seed transcription factor mutants *lec1-2*, and *fus3-3*, noticeable levels of HSP17.4 was present and when the same construct was transformed in these backgrounds, the seeds had better GUS staining in comparison to *abi3-6* mutant. Interestingly, the heat stressed embryos from these *HSP17.4*:GUS transformed in mutant backgrounds stained evenly and throughout for GUS thus clearly indicating the independent regulation of small HSPs during stress and development (Wehmeyer and Vieling 2000). In addition to this ABI3 has been shown to regulate the developmental expression of HsfA9 which is a heat shock transcription factor with unique expression in later seed development stages (Kotak et al. 2007). Experiments reveal the negligible accumulation of HsfA9 mRNA and protein alongwith other seed specific small HSPs (HSP17.4-CI, HSP17.7-CII) in ABI3 knockout mutant lines which was overcome by ABI3 overexpression in transgenic plants. ABI3 was also shown to activate the HsfA9 promoter while, HSfA9 could activate the promoters of few small HSP genes thus clearly establishing HsfA9 as a specialized member of the heat shock transcription factor family regulating developmental expression of small HSPs during seed maturation (Kotak et al. 2007) (Fig. 1.2).

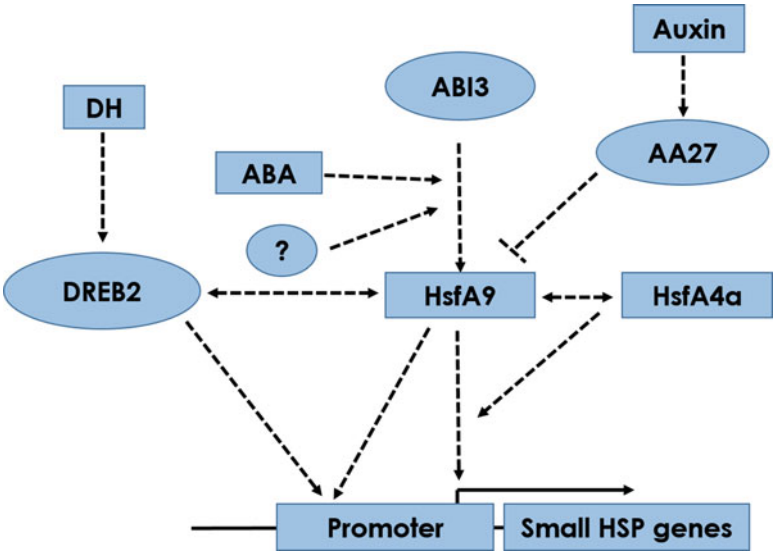

Fig. 1.2 Proposed model for developmental regulation of small HSPs in plants. HsfA9 is centrally positioned as it has been shown to be developmentally regulated by ABI3, a seed specific transcription factor and also is co-regulated by other transcription factors HsfA4a and DREB2. HsfA9, DREB2 and HsfA4a play important role both individually as well as in co-ordination for the regulation of downstream small HSP genes. Phytohormones, ABA and Auxin are also implicated for this developmental regulation of HSfA9 positively and negatively respectively

1.4 Concerted Expression of Small HSPs with Other Seed Storage Proteins

Seed development is a complex process which involves the accumulation of various macromolecules like carbohydrates, lipids, mRNAs and proteins in the seeds. All these components play important roles in preparing the seed for surviving desiccation and finally to complete germination successfully. Small HSP mRNAs are often expressed with some other seed specific protein mRNAs like LEA (Late Embryogenesis Abundant) proteins. Studies identified a small HSP (*HaHSP17.6*) and two LEA protein (D-113 and Emb-1) like mRNAs from sunflower seeds. These mRNAs were found to have similar expression profiles and the highest expression was noticed in mid maturation stage of embryos and dry seeds which was independent of the stress. Coordinated transcript accrual of small HSP with LEA proteins indicates the presence of shared transcription regulatory elements in their upstream regions (Almoguera and Jordano 1992). Interestingly enough in yeast a previously known small HSP (HSP12) was reclassified as a LEA protein. This study demonstrated that HSP12 accumulates in yeast cells during nutrient limited stationary phase when yeast cells are preparing for sporulation which is akin to seed maturation stage in plants when LEA protein synthesis takes place. Detailed amino acid sequence homologies and the hydropathy

plots also put forth another evidence for the reclassification (Mtwisha et al. 1998). Pea mitochondrial proteins, LEAM and HSP22 are also an example of the concerted expression of these two groups of proteins during seed development, desiccation tolerance and germination (Bardel et al. 2002; Macherel et al. 2007). Beech seeds stored for 1–8 years at −10 °C revealed the presence of dehydrin and dehydrin like proteins in addition to the LEA proteins and small HSP of 22 kDa. Dehydrins were found to express in both cotyledons and embryonic axes of dry stored seeds. In addition to dehydrins, small HSPs which might be induced by the oxidative stress and ROS generation during long term storage of beech seeds might function by decreasing the intracellular ROS and are positively correlated with germinability of stored beech seeds (Arrigo 1998; Kalemba and Pukacka 2008).

1.5 Small HSPs: Key Players in Acquisition of Seed Desiccation Tolerance

Small HSPs form a remarkable group of proteins and function in varied cellular processes. In addition to their obvious role in heat shock (although not all small HSPs have a heat induced expression) they are also synthesized during somatic embryogenesis, microsporogenesis, pollen formation and seed maturation (Bouchard 1990; Bouchard and Walden 1990; Zarsky et al. 1995; Vierling 1997; Coca et al. 1994; DeRocher and Vierling 1994). Small HSPs are known to function as molecular chaperones and their expression across abiotic stresses and various physiological stages makes them ideal candidates to counteract cellular damage caused by these conditions. Certain small HSPs have been shown to be induced in water stress and the expression of small HSPs during final stages of seed development and maturation highlights their importance in acquisition of desiccation tolerance (Almoguera et al. 1993; Coca et al. 1994; DeRocher and Vierling et al. 1994). Small HSP expression in pollen, in seed desiccation stages and in storage organs argue for their underlying unified role during quiescent stages of plant development marked with a decrease in water content and suppression of metabolism. All these stages are characterised by development of desiccation tolerance and are associated with accumulation of oligosaccharides, osmolytes and unique proteins like the late embryogenesis proteins (LEAs) and heat shock proteins (HSPs) (Hoekstra et al. 2001). Studies have suggested that during such desiccation events the cells form a glassy matrix consisting of soluble sugars which immobilize macromolecules offering protection to membranes and proteins (Leopold et al. 1994; Crowe et al. 1997; Bernal-Lugo and Leopold 1998; Buitink et al. 1998). Small HSPs are also hypothesized to play a role in glassy matrix formation coincident with their presence in late embryo maturation (Wehmeyer and Vieling 2000; Kalemba and Pukacka 2008). As a mechanism of action small HSPs are known to hold proteins under denaturing conditions and prevent their irreversible aggregation in addition to assisting in protein folding and helping with intracellular transport (Waters et al. 1996; Lee et al. 1997; Hendrick and Hartl 1995; Haslbeck et al. 2005).

1.6 Small HSPs Maintain Seed Viability During Aging

Recent studies have suggested the role of small HSPs in providing seed vigor and germinability under stress conditions. Transgenic plants overexpressing small HSPs have been reported to display improved stress tolerance such as thermotolerance (Perez et al. 2009; Sanmiya et al. 2004; Zhou et al. 2012), osmotic stress tolerance (Sun et al. 2001), chilling tolerance (Guo et al. 2007), and seed longevity (Prieto-Dapena et al. 2006; Kaur et al. 2015). In contrast, transgenic plants with reduced small HSP levels were less tolerant to thermal shock (Charng et al. 2006) and are susceptible to pathogens (Maimbo et al. 2007).

Studies on *Nelumbo nucifera* have revealed astonishing seed viability of more than 1300 years (Shen-Miller et al. 1995) which was attributed to the presence of several thermostable proteins including heat shock proteins (Shen-Miller et al. 2013). Ectopic expression of *NnHSP17.5* in Arabidopsis resulted in increased seed vigor under artificial aging conditions, where transgenic seeds showed remarkable germination. Interestingly, ectopic expression of *NnHSP17.5* in Arabidopsis resulted in increased SOD (superoxide dismutase) activity, a ROS scavenging enzyme, under artificial aging conditions, suggesting that small HSPs might play important roles in germination vigor by limiting free radical induced cellular damage (Zhou et al. 2012).

The presence of increased small HSP protein levels during initial stages of germination (3–4 DPI), indicates the role of small HSPs in rehydration and early seed establishment contributing to seed vigor (Wehmeyer et al. 1996; Sun et al. 2001; Zhou et al. 2012; Kaur et al. 2015; Koo et al. 2015). Abundance of small HSPs in dry seeds, during storage and germination was reported in pea (DeRocher and Vierling 1995), sunflower (Coca et al. 1994), Arabidopsis (Wehmeyer et al. 1996; Sun et al. 2001, 2002), *Nelumbo* (Zhou et al. 2012) and rice (Kaur et al. 2015) supporting the suggested function of small HSPs in seed vigor and viability.

Artificial aging reduces seed vigour by damaging proteins via carbonylation and reducing translation efficiency by damaging several components of protein translation machinery (Rajjou et al. 2008). Interestingly, HSPs were proven to play a protective role during protein carbonylation (Cabiscol et al. 2002) and in translation (Basha et al. 2004) thereby providing seed vigor and better germinability after aging. Beech (*Fagus sylvatica* L.) seeds which were aged at −10°C for 8 years showed increase in the levels of 22 kDa small HSP as a factor of time (Kalemba and Pukacka 2008). This accumulation of small HSPs during natural or artificial seed aging suggests protective roles of small HSPs during long term seed storage. A proteomic analysis of 6 day CDT (Controlled deterioration treatment) rice seeds identified small HSP18.2 to be induced after treatment. Arabidopsis seeds with seed specific overexpression of rice small HSP, *OsHSP18.2* displayed improved seed viability under CDT. The remarkable germination in OsHSP18.2 overexpressing lines correlated well with the reduced ROS levels in transgenic seeds as compared to the control (Kaur et al. 2015).

1.7 Small HSPs Impart Vigor and Better Germinability to Seeds Under Stress

Recent studies have demonstrated that constitutive overexpression of small HSPs not only improves germination under aging but also improves germination under stress conditions. Zou et al. (2012) have demonstrated that overexpression of *OsHSP17.0* and *OsHSP23.7* in rice resulted in improved seed vigor and seeds were able to germinate better under stress conditions. Transgenic rice seeds showed remarkable difference in the germination percentage under osmotic stress and salt stress showing reduced accumulation of MDA and electrolyte leakage, increased root/shoot length and increased proline content compared to wildtype seedlings. Increase in the germination vigor in rice seeds expressing *OsHSP17.0* and *OsHSP23.7* was attributed to reduction in membrane damage and increase in the protective molecules such as proline. Wheat chloroplastic small HSP26 when ectopically expressed in Arabidopsis resulted in increased seed vigor. Transgenic Arabidopsis seeds expressing *TaHSP26* were able to germinate and reach maturity even under continuous heat stress of 35 °C. Antisense plants showed reduced heat tolerance even to non-lethal heat stress providing a very strong evidence for the role of small HSPs in providing vigor to the seeds under stress conditions (Chauhan et al. 2012).

Ectopic expression of *LimHSP16.45* from David Lily (*Lilium davidii*) in Arabidopsis resulted in increased seed germination vigor under stress conditions. Over expression lines were able to germinate under heat stress showing germination upto 90 %, whereas wild type showed germination rates less than 60 %. *LimHSP16.45* overexpression in Arabidopsis also resulted in better germination in salt stress conditions showing 90–100 % germination, while wildtype showed only 70–80 % germination rate. Under oxidative stress overexpression lines showed better root growth in terms of root length whereas wildtype seedlings showed reduced root length. Improved seed vigor in the overexpression lines was associated with the increase in the levels of SOD and Catalase activities in transgenic seeds which might be supported by the chaperone activity of small HSPs (Mu et al. 2013). This shows that small HSP play a significant role in maintaining cell viability under stress conditions thereby improving seed vigor and viability. Increase in seed vigor and their ability to germinate under various stress conditions was demonstrated with the heterologous expression of *OsHSP18.2* in Arabidopsis. Transgenic Arabidopsis seeds showed improved germination under heat, dehydration and salt stress compared to controls. Strong evidence suggest that OsHSP18.2 is an aging responsive protein and plays a very important role in maintaining seed vigor and longevity. It also plays a crucial role in seedling emergence by protecting proteins from structural damage and restricting ROS accumulation (Kaur et al. 2015).

In tobacco light is one of the important factors that help in breaking seed dormancy. Light dependency of tobacco seeds was alleviated when *NtHSP18.2,*

NtHSP18.3 and *NtHSP17.6* small HSPs, were overexpressed. A similar light independent germination was also observed when seeds were subjected to heat stress prior to germination (Koo et al. 2015). These evidences suggest the small HSPs might also be involved in breaking dormancy through mechanisms still not known.

1.8 Conclusion

The small HSP class of heat shock proteins has been very well characterized for their heat shock related roles; however, their interesting accumulation patterns during various stages of development in plants e.g. pollen and seed development and seed germination has led to intriguing investigations into their diverse roles and functional capabilities. These developmental stages share a unifying underlying physiological state of desiccation or loss of cellular water (during pollen and seed development) or rehydration (during seed germination). Hence, the associated accumulation of small HSPs during these stages may possibly prepare these plant organs to face the imminent loss of water. Moreover, the chaperone function of small HSPs might also contribute by preventing the irreversible denaturation of important cellular proteins during water deficit conditions as encountered in these stages of plant life cycle. Small HSPs have also been implicated in the formation of glassy matrix in the cells during late seed maturation stages along with other important seed specific proteins like LEA proteins. Thus, the small HSPs are a unique class of heat shock proteins which are equally important for their roles in protection towards heat stress or other environmental stresses and in plant development and various key stages of plant life cycle.

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Chapter 2
Plant Small Heat Shock Proteins and Its Interactions with Biotic Stress

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Abstract Small heat shock proteins were first identified during heat shock stress, but currently have been often associated to plant biotic stresses. Considered stress defense proteins, HSP20s functions are especially related to interact with unfolded model substrate proteins, in ATP-independent manner, and keep them in a folding-competent state for subsequent refolding. These proteins have been reported to serve a role in plant-response against the most important crop phytopathogens in the world, such as nematodes and fungi. Their activation role during biotic stress is not completely elucidated, but some researches have demonstrated that in some occurrences of plant response to biotic stresses, there is a crosstalk between the abiotic stress responses. Some genetic evidence has revealed that the chaperones play a critical role in plant immunity. One hypothesis is the chaperone activity can provide the stability and accumulation of R proteins, and thus for the entire defense signaling cascade coordination. However, the researches about this issue still need to better elucidate HSP20 pathways and function in plant biotic stress.

Keywords HSP family • HSP20 • Plant defense mechanism • Pathogens

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Abbreviations

ACD	conserved α-crystallin domain
Avr gene	avirulence gene
bp	base pairs
ESTs	expressed sequence tags ETI effector-triggered immunity
GUS	β-glucuronidase
HMM	hidden markov model
HSE	heat shock element
HSFs	heat transcription factors
HSP	heat shock protein
MAMPs	microbe-associated molecular patterns
NB-ARC- LRR	nucleotide binding site-leucine-rich regions
NB-LRR	nucleotide binding domain and leucine-rich repeat
NIL-R	resistance near-isogenic lines
NIL-S	susceptible near-isogenic lines
PAMPs	pathogen associated molecular patterns
PCR	polymerase chain reaction
PRRs	plant pattern-recognition receptors
PTI	Physical association of pattern-triggered immunity
QTL	Quantitative trait locus
RdRp	RNA-dependent RNA polymerase
R genes	resistance genes
RLKs	receptor-like kinases
RLPs	receptor-like proteins
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SCN	soybean cyst nematode
sHSPs	small heat shock proteins
SSR	simple sequence repeats
Y2H	yeast two-hybrid

2.1 Introduction

In the course of evolution, organisms have developed strategies to overcome the different types of abiotic and biotic stress, including a network of proteins to protect cells (Shanker and Venkateswarlu 2011; Richter et al. 2010). An important group of these stress responsive proteins is composed by chaperones, or heat shock proteins (HSP), and their presence has been demonstrated in all living organisms (Tkáčová and Angelovičová 2012). HSPs comprise several families classified according to their molecular weight: HSP100, HSP90, HSP70/DnaK and HSP60/GroE and HSP20 or small heat shock proteins (sHSPs), representing sizes of 15–42 kDa (Waters 2013).

During their lifetime, due to their sessile nature, plants are continually exposed to adverse effects but may supplant them through the evolution of various morphological and physiological mechanisms that enable them to survive stress (Al-Whaibi 2011). In higher plants, HSP20 are encoded by nuclear multigenic families and have undergone great functional diversification (Waters et al. 1996). Plants usually contain around 20 HSP20, or more, but this number can be four times lower in animal organisms (Waters et al. 1996; Franck et al. 2004).

Furthermore, it is noteworthy that no HSP20 proteins located in plant organelles have homologs in animals, fungi, or even in green algae (Waters et al. 2008); these the HSP20 appear to be localized only in the cytoplasm/nucleus while plants have several subfamilies, which are distributed in different cellular compartments. In *Arabidopsis*, 19 genes encoding HSP20 were classified into 12 subfamilies, according their cellular localization and phylogeny (Scharf et al. 2001), while 36 Hsp20 were identified in *Populus trichocarpa* (Waters et al. 2008), 23 in *Oryza sativa* (Sarkar et al. 2009), 51 in *Glycine max* (Lopes-Caitar et al. 2013), 35 in *Capsicum annuum* L., 27 in *Triticum aestivum* and 13 in *Hordeum vulagre* (Pandey et al. 2014).

HSP20 are often the most abundant plant stress responsive class among the heat shock proteins group (Heckathorn et al. 1999). They also have been largely studied in human health and diseases (Bakthisaran et al. 2015). The primary structures of HSP20s are characterized by a conserved α-crystallin domain (ACD) from 80 to 100 amino acids at the C-terminal region (Cashikar et al. 2005). This domain is divided into two consensus regions designated I and II, separated by a hydrophobic region of variable size. The consensus region I (~27 amino acids) N-terminal constitutes conserved sequence Pro-$X_{(14)}$-Gly-Val-Leu; and the consensus region II (~29 amino acids) C-terminal, has the conserved sequence Pro-$X_{(14)}$/Val/Leu/Ile-Val/Leu/Ile. ACD is preceded by an N-terminal variable size region and considerable diversity in their sequence (Cashikar et al. 2005; Waters et al. 2008). Within HSP20-ACD domain there is a typical secondary structure formed, usually with the formation 6–8 β-sheet configuration. The β settings are numbered along the amino acids chain in the amino-caboxiterminal sense, being found within the ACD, usually the β2 chains β9 (Siddique et al. 2008) (Fig. 2.1).

Each class of HSPs has a characteristic function upon specific spectrum (broad or narrow) of substrate proteins in assisting their folding, refolding, oligomeric assembly, translocation, and/or degradation (Hartl et al. 2011). The HSP20 are known to act as ATP-independent molecular chaperones that bind to the newly synthesized proteins and denatured proteins to prevent them from forming irreversible aggregations, under stress conditions, also can be called "holdase" (Zhang et al. 2015b). However, they have been classified within the group of molecular chaperones for the ability to recognize and bind to denatured proteins or inappropriate folding and thus prevent improper interactions that lead to irreversible aggregate formation, precipitation, and degradation thereof by proteases (Sun et al. 2002).

The HSP20s are ubiquitin that form large oligomeric hetero-complexes ranging in size 200–800 kDa and have also been found to suppress protein aggregation in an ATP-independent manner, stabilize stress-damaged cell membranes, tag denatured proteins aggregates, or avoid inappropriate folding, to further action of proteases in

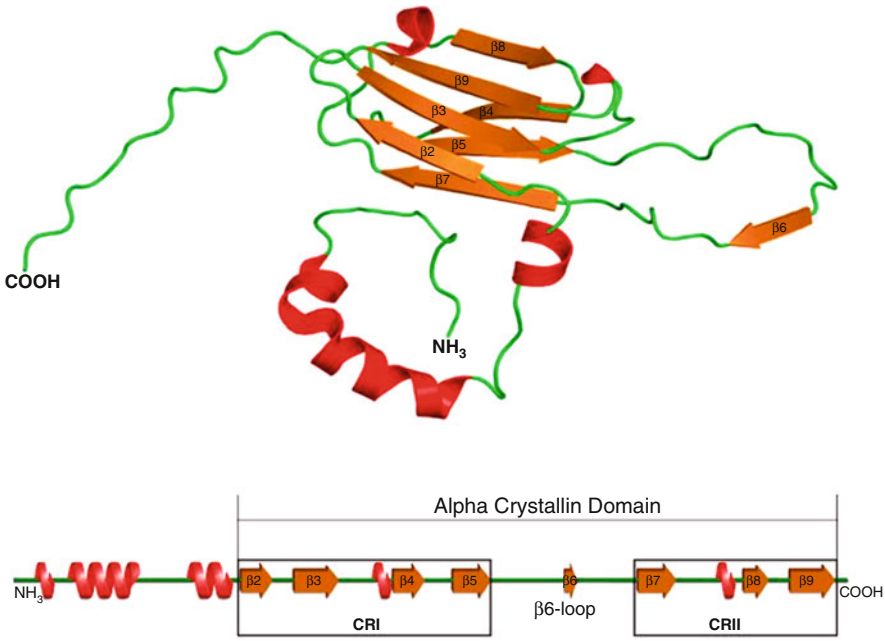

Fig. 2.1 Structural organization of a typical sHSP protein. The picture shows the X-ray structure of a Triticum aestivum sHSP dimer (protein data bank in Europe: 1GME) and primary structure (protein data bank PDBsum: 1GME) of the protein that are constituted by C-terminal region, N-terminal region and a α-crystallin domain (ACD) containing the conserved regions I (CRI) and II (CRII) and the β6-loop (Modified from Bondino et al. 2012)

both, prokaryotic and eukaryotic cells (Fu 2014). The main HSP20s activity is to interact, while still possible, with unfolded model substrate proteins. HSP20 keeps another protein in a folding-competent shape for subsequent refolding, by ATP-dependent chaperones as HSP70s and HSP100s, or labels them and allows the proteinases action, if necessary (Al-Whaibi 2011; Fu 2014).

In this chapter, we will discuss how plant small heat shock proteins are related to response against biotic stress and how the knowledge about its function and involvement in response to different types of stress can be explored as an important tool in agricultural sciences.

2.2 Plant HSP Family

The plant heat shock proteins have several functions related to stress response and it can be verified by each subclass. The HSP100 family usually located in the cytosol, mitochondria and chloroplast, exhibit weight around 97–114 kDa (Singla et al. 1998; Park and Seo 2015). This protein family has been detected in various plant species, such as in Glycine max, where the HSP103 kDa protein was present in soybean cell subjected to heat stress. Similarly, the presence of the protein HSP100

and 110 was detected in *Nicotiana tabacum* under heat stress (Barnett et al. 1980). Besides these species, already was noted the HSP100 protein in *Gossypium hirsutum*, *Gossypium hirsutum*, *Opuntia ficus indiea*, *Prosopis chilensis*, *Saccharum. Officinarum*, *Secale cereate*, *Triticum aestivum*, *T. aestivum*, *T. durum*, *Vigna radiate* and *Zea mays* (Singla et al. 1998).

In the model plant *Arabidopsis thaliana*, the family HSP90 includes 7 members: At*Hsp*90-1 to At*Hsp*90-4 genes, which constitute the cytoplasmic subfamily, AtHsp90-5, AtHsp90-6, and AtHsp90-7 genes, which are predicted to be within the plastidial, mitochondrial, and endoplasmic reticulum compartments, respectively (Krishna, 2001). Therefore, HSP90 are located in several cellular compartments, such as cytosol, mitochondria, chloroplast, nucleus and endoplasmic reticulum (Park and Seo 2015). Additionally, HSP90 can be found in *Chlamydomonas reinhardtii* and *Oryza sativa* species (Chen et al. 2006).

Regarding HSP70, *A. thaliana* genome contains 12 subfamilies, with five belonging to cytosol compartment (Hsc70-1, Hsc70-2, Hsc70-3, Hsp70 and Hsp70b), three belonging to endoplasmic reticulum lumen (BiP-1, BiP-2 and BiP-3), two belonging to mitochondrion matrix (mtHsc70-1 and mtHsc70-2), and two belonging to plastid stroma (cpHsc70-1 and cpHsc70-2) (Sung et al. 2001). The HSP70 family was also detected in other plants species, such as *Capsicum annuum L., Cucurbita maxima, Cucumis sativus, Nicotiana tabacum, Gossypium hirsutum, Populus trichocarpa, Solanum lycopersicum, Pisum sativum, Pisum sativum, Theobroma cacao, Hevea brasiliensis, Spinacia oleracea,* and *Oryza sativa* (Guo et al. 2014).

A phylogenetic analysis of HSP60 gene family from *Populus trichocarpa*, *A. thaliana* and *Oryza sativa*, suggested that these plant species contend approximately 28, 18 and 20 HSP60, respectively, categorized into four subfamilies Zhang et al. (2015a). This group is cytosol-localized Cpn60 (18 genes), mitochondrion-localized Hsp60 (3 genes), and chloroplast-localized Cpn60-a (4 genes) and Cpn60-b (3 genes) (Zhang et al. 2015a). Additionally, the HSP60 family was also identified in several other plant species, such as Glycine max, *Medicago truncatula, Physcomitrella patens, Ricinus communis, Solanum tuberosum, Sorghum bicolor, Triticum aestivum, Vitis vinífera* and *Zea mays* (Ratheesh Kumar et al. 2012).

Like other proteins, HSP20 was found in several plant species, such as *Arabidopsis thaliana, Petunia hybrid, Pisum sativium, Triticum aestivum, Zea mays, Chenopodium rubrum, Glycine max, Daucus carota, Helianthus annuus, Lycopericoscon esculentum, Medicago sativa, Ipomea nil* and *Lilium longijlorum* (Waters 1995).

The genome-wide analysis of the HSP20 protein family was performed with at least 6 species: *Oryza sativa*, *G. max*, *T. aestivum*, *Hordeum vulgare* L., *Capsicum annuum* and *Arabidopsis*. (Guo et al. 2015; Lopes-Caitar et al. 2013; Pandey et al. 2015; Sarkar et al. 2009; Scharf et al. 2001).

Pandey et al. (2015) reported in their studies HSP20 genes in wheat (TaHSP20) and barley (HvHSP20). Using Hidden Markov Model (HMM) and Blast algorithm, the authors identified 27 newly TaHSP20 candidate genes in wheat and 13 HvHSP20 in barley. Interestingly, the HSP20 protein predicted were located in both mitochondria and the nucleus, however, a large number of TaHSP20 and HVHSP20