Chapter 14

Role of Heat Shock Proteins in Improving Heat Stress Tolerance in Crop Plants

Palakolanu Sudhakar Reddy, Thammineni Chakradhar, Ramesha A. Reddy, Rahul B. Nitnavare, Srikrishna Mahanty, and Malireddy K. Reddy

Abstract High temperature response (HTR) or heat stress response (HSR) is a highly conserved phenomenon, which involves complex networks among different crop species. Heat stress usually results in protein dysfunction by improper folding of its linear amino acid chains to non-native proteins. This leads to unfavourable interactions and subsequent protein aggregation. To tackle this, plants have developed molecular chaperone machinery to maintain high quality proteins in the cell. This is governed by increasing the level of pre-existing molecular chaperones and by expressing additional chaperones through signalling mechanism. Dissecting the molecular mechanism by which plants counter heat stress and identification of important molecules involved are of high priority. This could help in the development of plants with improved heat stress tolerance through advanced genomics and genetic engineering approaches. Owing to this reason molecular chaperones/Heat shock proteins (Hsps) are considered as potential candidates to address the issue of heat stress. In this chapter, recent progress on systematic analyses of heat shock proteins, their classification and role in plant response to heat stress along with an overview of genomic and transgenic approaches to overcome the issue, are summarized.

Keywords Heat shock element • Heat shock factors • Heat shock proteins • Heat shock response • Heat stress

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P.S. Reddy (✉)
Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110 067, India

Crop Physiology, Dryland Cereals, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502 324, Telangana, India
e-mail: p.sudhakarreddy@cgiar.org; palakolanusreddy@gmail.com

T. Chakradhar • R.A. Reddy • S. Mahanty • M.K. Reddy
Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110 067, India

R.B. Nitnavare
Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Pune, Maharashtra 411 027, India

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Abbreviations

HSE	heat-shock element
HSF	heat shock factor
HSPs	heat shock proteins
HSR	heat stress response
HTR	high temperature response

14.1 Introduction

Global warming, along with the inevitable climatic changes is estimated to affect the global temperatures by an average of 3–5 °C increase in near future (Kerr 2007). With this predicted rise in temperature, heat stress is gaining as the trait of importance to breed for climate resilient crops. Prolonged incidents of heat waves caused by frequent fluctuations in daily and seasonal temperatures pose a serious challenge for agricultural production worldwide, affecting plant growth and yield with annual loss estimated up to billions of dollars (Mittler et al. 2012). Hence increasing crop productivity in view of escalating population and diminishing arable land and natural resources has become a matter of urgency than merely a research theme. To overcome such heat stress conditions, plants have developed several tolerance mechanisms. To understand the molecular basis of the tolerance mechanisms, knowledge of modern tools in molecular and genetic engineering is essential. Many abiotic stress-inducible genes were dissected and their functions are precisely characterized using functional genomics approaches. Another significant progress made in understanding this complex trait of heat tolerance is completion of the genome sequence information in major crop species including rice, maize, sorghum etc. This information has allowed identification and monitoring of transcript profiling for all the predicted genes at a single shot by either microarray or RNA sequencing approaches. The availability of vast amount of genome data has also enabled the identification of potential *cis*-regulatory elements and *trans*-factors.

Heat stress usually effects in protein dysfunction by improper folding of its linear peptide chains to non-native proteins leading to unfavourable interactions and subsequent protein aggregations (Moriwaki et al. 1999). Under stress conditions not only the nascent polypeptides face error-prone folding but also a large portion of the folded proteins gets partially or completely denatured and re-enter the protein quality control machinery assisted by molecular chaperones (Hebert and Molinari 2007). Nature has developed efficient molecular chaperon machinery in plants to maintain high quality proteins in the cells by increasing the level of pre-existing molecular chaperones and by expressing additional chaperones through signalling mechanism (Buchberger et al. 2010). Many proteins in a living cell will not fold properly without the assistance of molecular chaperones (Buchberger et al. 2010). Heat shock proteins (Hsps) are class of molecular chaperones that play an essential role in preserving cellular functions under stressful conditions. All living organisms are equipped with evolutionarily conserved Hsps to encounter sudden climate changes of nature. Hsps have broad range of functions ranging from the prevention of protein aggregation, refolding of misfolded proteins, and degradation of unstable proteins and dissolution of protein complexes, besides some act as transcription factors. Based on their differences in molecular weight, Hsps are classified into five sub-classes: Hsp100, Hsp90, Hsp70, Hsp60 and low molecular weight Hsps or small sHsps (Wang et al. 2004). Various members of Hsps have been cloned and functionally characterized and some of these have resulted in developing transgenic plants showing tolerance to various abiotic stresses (Lavania et al. 2015). Hsps and heat shock transcription factors (Hsfs) play a crucial role in heat stress tolerance during flowering and grain filling stages as evident in several examples (Waters 2003; Bita and Gerats 2013). However, detailed characterization and the role of plant Hsps as chaperones have been investigated only in a few model plants. The mechanisms of Hsps underlying abiotic stress adaptation in plants and the pivotal role of molecular chaperons will be discussed in the light of recent developments in genomics and genetic engineering approaches. The information and list of the transgenic plants developed for heat stress tolerance are discussed under the following sections.

14.2 Heat Shock Proteins (Hsps)

Heat stress disturbs cellular homeostasis, causes severe growth retardation effecting plant development, and become more vulnerable if occurs during flowering. Higher plants are unable to cope up with the extended exposure to temperatures above 45 °C (Herrenkohl and Politch 1978). The loss of biological activity of proteins upon high temperature stress may be due to aggregation and/or protein misfolding (Grover et al. 2013). The stress-induced accumulation of aggregated and mis-folded proteins is irreversible and deleterious to the cell functioning. To balance the homeostasis of cellular proteins under heat stress, plant cell upregulates several heat inducible genes, commonly referred as “heat shock genes” (HSGs), which encode Hsps that makes plants survival under high temperature (Chang et al. 2007a, b). A wide range of proteins have been reported to possess chaperone activity (Lindquist and Craig 1988). These are also called as molecular chaperones because with the help of several other proteins, commonly called as co-chaperones, they bind to partially folded or denatured proteins and prevent them from self-aggregation or promote their proper folding both in ATP dependent and independent manner. However, during their function they neither covalently bind to the substrate proteins nor form the

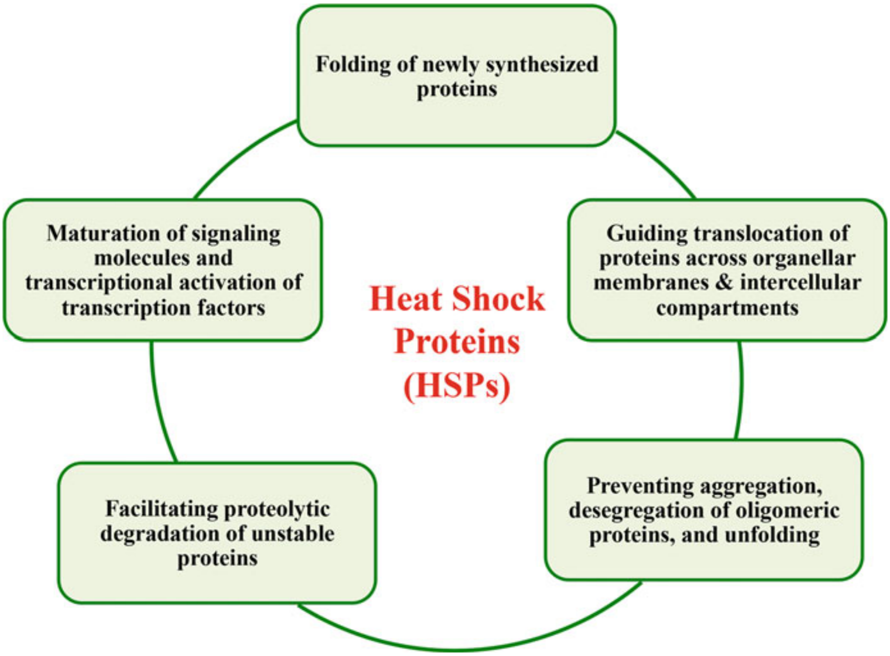

Fig. 14.1 Diverse functions of Heat shock proteins (Hsps)

part of the final product. These Hsps are broadly divided into two major families i.e., low and large molecular weight Hsps which again subdivided into five major classes based on the sizes of the corresponding proteins such as Hsp100/Clp, Hsp90, Hsp70, Hsp60/chaperonin and sHsps (Wang et al. 2004). Under normal conditions they perform many cellular functions such as (1) folding or assisting folding of newly synthesized proteins (Hsp70, Hsp60), (2) guiding translocation of proteins across organellar membranes and between intercellular compartments (Hsp70) (3) preventing aggregation, desegregation of oligomeric proteins, and unfolding (Hsp70, Hsp100, Hsp90, small Hsps) (4) facilitating proteolytic degradation of unstable proteins (Hsp70, Hsp100), (5) maturation of signaling molecules, signal transduction and transcriptional activation of transcription factors (Hsp70, Hsp90) (Driedonks et al. 2015) (Fig. 14.1). Many plant biotechnologists characterized the transcription and translation of Hsps in response to heat stress in different plant species (*Arabidopsis*, rice, wheat, tomato and maize) and their involvement in regulating thermotolerance has been established through forward and reverse genetic approaches (Lavania et al. 2015; Driedonks et al. 2015; Usman et al. 2014).

14.3 Small Heat Shock Proteins (sHsps)

Among five conserved families of Hsps, the sHsps are found to be most prevalent in plants and their expression can be increased up to 200 folds under heat stress (Wang et al. 2004). sHsps range in size from 10 to 42 kDa and share a conserved C-terminal domain that is common to all eukaryotic organisms (Waters et al. 1996). sHsps family shows diversity with respect to sequence similarity, cellular location and functions (Reddy et al. 2014; Reddy et al. 2015). In plants six different multi gene families that encode for sHsp proteins are localized in compartments like cytosol, endoplasmic reticulum (ER), mitochondria and chloroplast (Reddy et al. 2014). sHsps do not actively participate in refolding of non-native proteins (Veinger et al. 1998; Lee and Vierling 2000). They possess a high capacity of binding to non-native proteins, through hydrophobic interaction (Reddy et al. 2000). sHsps perhaps prevent non-native aggregation, thereby facilitating subsequent refolding through ATP-dependent chaperones such as the DnaK system or ClpB/DnaK.

The abundance of sHsps in plants and their functional characteristics of binding and stabilizing denatured proteins suggest that sHsps play an important role in plant-acquired stress tolerance (Sun et al. 2002). To support this, transgenic carrot cell lines with *Hsp17.7* gene under the control of CaMV35s promoter were developed, which resulted in enhanced survival of cell lines and plants at high temperature (48 °C) (Sun et al. 2002). The transformed seedlings with Class I sHsps showed higher cotyledon opening rate in tobacco plant (Park et al. 2002). In contrary, seedlings raised with the antisense construct in this experiment showed increased sensitivity to heat shock indicating the role of sHsps in seed germination at high temperatures. Transgenic rice plants over expressing with *OsHsp17.7* gene showed increased thermo tolerance as well as increased resistance to UV-B irradiation (Murakami et al. 2004). Tomato *mtLeHsp* gene when over expressed in tobacco-conferred thermotolerance up to 48 °C compared to their counter transgenics developed through antisense construct of the same gene (Sanmiya et al. 2004). Transgenic *Arabidopsis* plants over expressing with *NnHsp17.5, RcHsp17.8, ZmHsp22, ScHsp26* and *LdHsp16.45* showed heat tolerance to varied extents (Rhoads et al. 2005; Jiang et al. 2009; Sun et al. 2012; Zhou et al. 2012). Transgenic *Arabidopsis* plants over expressed with *WsHsp26* was tolerant under continuous high temperature and produced bold seeds under high temperature, having higher germination rate than wild type (Mu et al. 2013). In *Arabidopsis*, over expression of *RcHsp17.8* enhanced SOD activity (Jiang et al. 2009) whereas over expression studies of *ZmHsp16.9* in tobacco enhanced POD, CAT and SOD activity indicating the role of sHsps in oxidative stress tolerance (Chauhan et al. 2012). Altogether, it may be hypothesized that the sHsp proteins positively affect thermotolerance by maintaining the threshold levels of ROS scavenging enzymes, that could initiate the signaling pathway of thermotolerance (Driedonks et al. 2015). The updated list of the transgenic plants developed for sHsps is listed in Table 14.1.

Table 14.1 Transgenic plants made by means of dissimilar Hsp genes for heat stress tolerance

S. No	Gene	Source	Transgenic	Promoter	Reference
1	*Hsp17.7*	*D. carota*	*D. carota*	35s	Malik et al. (1999)
2	*sHsp17.7*	*O. sativa*	*O. sativa*	35s	Murakami et al. (2004)
3	*sHsp17.7*	*O. sativa*	*O. sativa*	35s	Sato and Yokoya (2008)
4	*Hsp17.5*	*N. nucifera*	*A. thaliana*	35s	Zhou et al. (2012)
5	*Hsp17.8*	*R. chinensis*	*A. thaliana*	35s	Jiang et al. (2009)
6	*Hsp17.8*	*A. thaliana*	*L. sativa*	35s	Kim et al. (2013)
7	*Hsp17/Hsp23*	*O. sativa*	*O. sativa*	35s	Zou et al. (2012)
8	*Hsp17.9*	*P. mume*	*A. thaliana*	35s	Wang et al. (2016)
9	*Tlhs1*	*N. tabacum*	*N. tabacum*	35s	Park and Hong (2002)
10	*mtsHsp*	*S. lycopersicon*	*N. tabacum*	35s	Sanmiya et al. (2004)
11	*Hsp21*	*S. lycopersicon*	*S. lycopersicum*	35s	Neta-Sharir et al. (2005)
12	*Hsp16.9*	*Z. mays*	*N. tabacum*	35s	Sun et al. (2012)
13	*Hsp16.45*	*L. davidii*	*A. thaliana*	35s	Mu et al. (2013)
14	*Hsp18*	*O. streptacantha*	*A. thaliana*	35s	Salas-Munoz et al. (2012)
15	*Hsp22*	*Z. mays*	*A. thaliana*	35s	Rhoads et al. (2005)
16	*Hsp23*	*M. sativa*	*A. stolonifera*	35s	Lee et al. (2015)
17	*Hsp23*	*M. sativa*	*F. arundinacea*	35s	Lee et al. (2012)
18	*Hsp24.4*	*M. acuminata*	*S. lycopersicum*	35s	Mahesh et al. (2013)
19	*Hsp26*	*O. sativa*	*F. arundinacea*	35S	Kim et al. (2012)
20	*Hsp26*	*S. cerevisiae*	*A. thaliana*	35s	Xue et al. (2010)
21	*Hsp26*	*T. aestivum*	*A. thaliana*	35s	Chauhan et al. (2012)
22	*ChlDnaJ/Hsp40*	*L. esculentum*	*L. esculentum*	35s	Kong et al. (2014)

(continued)

Table 14.1 (continued)

S. No	Gene	Source	Transgenic	Promoter	Reference
23	*DnaK/Hsp70*	*A. halophytica*	*N. tabacum*	35s	Ono et al. (2001)
24	*DnaK/Hsp70*	*A. halophytica*	*N. tabacum, O. sativa*	35s	Uchida et al. (2008)
25	*Hsp70*	*N. tabacum*	*N. tabacum*	35s	Cho and Choi (2009)
26	*Hsp70*	*T. harzianum*	*A. thaliana*	35s	Montero-Barrientos et al. (2010)
27	*mtHsp70*	*O. sativa*	*O. sativa*	35s	Qi et al. (2011)
28	*Hsp70*	*C. morifolium*	*A. thaliana*	35s	Song et al. (2014)
29	*Hsp70*	*B. campestris*	*N. tabacum*	35s	Wang et al. (2015)
30	*Hsp70*	*E. arundinaceus*	*Saccharum spp.*	Ubi2.3	Augustine et al. (2015b)
31	*Hsp70*	*A. thaliana*	*M. sativa*	35s	Ferradini et al. (2015)
32	*Hsp70*	*M. uniflorum*	*A. thaliana*	35s	Masand and Yadav (2016)
33	*Hsp90*	*G. max*	*A. thaliana*	35s	Xu et al. (2013)
34	*Hsp90.7*	*A. thaliana*	*A. thaliana*	35s	Chong et al. (2015)
35	*Hsp101*	*A. thaliana*	*A. thaliana*	35s	Queitsch et al. (2000)
36	*Hsp101*	*A. thaliana*	*O. sativa*	ZmUbi	Katiyar-Agarwal et al. (2003)
37	*Hsp101*	*O. sativa*	*N. tabacum*	35s	Chang et al. (2007a)

14.4 Heat Shock Protein 70 (Hsp70)

The second most evolutionarily conserved Hsp family in diverse organisms is Hsp70 (Boorstein et al. 1994). Hsp70's have two major functional domains, an ATPase domain of 44 kDa at the N-terminus and a 25 kDa peptide-binding domain at C-terminus and, are separated by small linker region (Reddy et al. 2010). The substrate-binding domain comprises of a sandwich of 2-four-stranded β-sheets, where the peptide-binding cleft resides. Another feature of plant Hsp70's is the presence of identifiable unique amino acid signature motif at the C-terminus that can be used to distinguish the protein's sub-cellular location. The EEVD motif indicates the cytosol-specific, HDEL for endoplasmic reticulum-specific;

PEGDVIDADFTDSK for plastid-specific and PEAEYEEAKK for mitochondrion-specific location of Hsp70 proteins (Reddy et al. 2010; Guy and Li 1998). Hsp70 class of proteins involved in many functions like controlling the biological activity of folded regulatory proteins, negative repressors of heat-shock factor (Hsf) mediated transcription. Some Hsp70's also exists in symbiosome membrane, which is known to play an important role in nodule development (He et al. 2008). The activity of Hsp70's can also be regulated by post-translational modifications (Napolitano et al. 1987) and by interaction with other co-chaperones (Santacruz et al. 1997). Hsp70's are also involved in protein import and translocation processes, and in facilitating the proteolytic degradation of unstable proteins by targeting the proteins to lysosomes or proteasomes (Hartl 1996). In addition to its general chaperone functions, Hsp70 also displays a regulatory role in other stress-associated gene expression (Lee and Schoffl 1996). Unfortunately, the role of Hsp70's in the modulation of signal transduction has not been studied in plants.

Hsp70's have been reported to be involved in ABA responses, redox signalling, chloroplast development, and protein translocation into chloroplasts and mitochondria and hence over expression of this class of chaperons leads to increased resistance against drought, high salt and heat stresses in plants (Lee et al. 2012). A halotolerant cynobacterial Hsp70/DnaK gene, when over expressed in tobacco and rice exhibited increased levels of anti-oxidant enzymes and enzymes involved in Calvin cycle conferring temperature and drought stress tolerance particularly during reproductive stage (Uchida et al. 2008). The over expression of Hsp70 from fungus *Trichoderma harzianum* in *A. thaliana* resulted in increased level of Na/H transporter (SOS1) and APX1 with decreased levels of Hsf and Hsp transcripts (Montero-Barrientos et al. 2010). Over expression of rice *mtHsp70* in rice resulted in lesser production of heat induced ROS, higher mitochondrial membrane potential and suppressed programmed cell death (Qi et al. 2011). Constitutive expression of a chrysanthemum Hsp70 in *A. thaliana* enhanced the tolerance against heat, drought and salinity stresses (Song et al. 2014). Hsp 70 from *E. arundinaceus* in sugarcane was shown tolerance to drought and salt stresses (Augustine et al. 2015a). Over expression of *B. campestris* Hsp70 in transgenic tobacco plant had shown heat stress tolerance by enhancing superoxide dismutase (SOD) and peroxidase (POD) activity, soluble sugar content and reduced electrical conductivity than control plant (Masand and Yadav 2016). Transgenic *A. thaliana* over expressing Hsp70 of *M. uniflorum* confers tolerance to multiple abiotic stresses and further shown the reduced levels of malondialdehyde (MDA), H_2O_2 and proteolytic activity. The transgenics have maintained the better shoot biomass, root length, relative water content and chlorophyll content during exposure to stresses relative to wild type plant (Chen et al. 2006). Other studies have found similar effects of Hsps on ROS scavenging proteins up on heat stress. The current status and updated list of the Hsp70 transgenic plants is given in Table 14.1.

14.5 Heat Shock Protein 90 (Hsp90)

Hsp90 family, which is highly, conserved molecular chaperones that are ubiquitously present in a wide range of organisms from prokaryotes to eukaryotes, except Archaea (Johnson and Brown 2009). In eukaryotic organisms, the cytosolic Hsp90 exists in two isoforms, inducible α-form and constitutive ß-form and at least one of these isoforms is functionally essential for the survival of the organism (Reddy et al. 2011). Due to slight variations in their relative molecular masses, these protein homologs have been represented by different names in literature (e.g. Hsp80, Hsp81, Hsp82, Hsp83, Hsp84, Hsp90 etc.). Amino acid sequence analysis of Hsp90 gene family can reveal their subcellular localization. This is possible due to presence of distinguishable amino acid signature motifs either at the N- or C-terminus region i.e. C-terminus MEEVD penta-peptide motif for cytosol-specific Hsp90 isoforms and C-terminus HDEL motif for endoplasmic reticulum-specific Hsp90 isoforms, whereas a characteristic N-terminus extension of signal peptide sequence for chloroplast and mitochondrion-specific Hsp90's (Pearl and Prodromou 2006). Hsp90 family predominantly occurs as a homodimer with three modular structural domains (Sangster and Queitsch 2005). The N-terminal domain contained the ATP-binding site responsible for the weak intrinsic ATPase activity of Hsp90. The middle domain, deliberated as a major site for client protein interaction, was connected to the N-terminal domain through a highly charged linker region. The C-terminal domain confined with the dimerization interface and a conserved C-terminal MEEVD motif, which was responsible for interaction with tetratricopeptide repeat (TPR) domain-containing co-chaperones. Hsp90's are constitutively present up to 1–2 % in cellular proteins; however, their expression is increased further by several folds on exposure to abiotic stresses mainly heat stress. Hsp90's are also considered as marker for morphological evolution (Sangster and Queitsch 2005). This suggests that Hsp90 functions as regulatory housekeeping protein as well as a molecular chaperone (Liu et al. 2006; Xu et al. 2013). Similar results were obtained during over expression of five Hsp90 genes of *Glycine max* in *A. thaliana.* Results obtained showed involvement of Hsp90 in different plant functions like higher biomass production, pod setting, reduction in lipid peroxidation and loss of chlorophyll under heat stress (Neuwald et al. 1999). The updated list of the transgenic plants developed for Hsp90 is presented in the Table 14.1.

14.6 Heat Shock Protein 100 (Hsp100)

The Hsp100/Clp are hexameric rings belonging to the large AAA ATPase super family with a broad spectrum of diverse functional properties (Agarwal et al. 2001; Keeler et al. 2000). Hsp100 was first described as components of the two-subunit bacterial Clp protease system, which consists of regulatory ATPase/chaperones (such as ClpA and ClpX) and proteolytic (ClpP) subunits. So far, Hsp100/Clp proteins have been reported in many plant species, such as *Arabidopsis*, soybean, tobacco, rice, maize, lima bean (*Phaseolus lunatus*) and wheat (Keeler et al. 2000; Adam et al. 2001; Schirmer et al. 1996). Hsp100 family is divided into two major classes and eight distinct subfamilies. Members of the first class (A-D) contain two nucleotide-binding domains (also called ATP-binding domains), whereas those in the second class (M, N, X, Y) have only one nucleotide-binding domain (Schirmer et al. 1994). In lima bean, Hsp100's are revealed to have expression in cytosol and chloroplasts when exposed to heat stress (Adam et al. 2001). Genetic evidence indicates a role for this family of proteins in thermo protection (Lee et al. 1994; Glover and Lindquist 1998). Contrasting to the regular chaperone function of preventing protein aggregation and misfolding, the Hsp100/Clp family has a functional role in protein disaggregation and/or protein degradation. The removal of non-functional but potentially harmful polypeptides arising from misfolding, denaturation or aggregation is important for the maintenance of cellular homeostasis. The mechanism for rescuing proteins from aggregation also involves the cooperation of another ATP-dependent chaperone system, the Hsp70. The Hsp100/Clp family solubilizes the aggregated protein and releases it in a state that can be refolded with the assistance of the Hsp70 system (Goloubinoff et al. 1999; Adam and Clarke 2002) Like many other Hsps/chaperones, Hsp100/Clp family chaperones are often constitutively expressed in plants, but their expression is developmentally regulated and is induced by different environmental assaults, such as heat, cold, dehydration and high salt or dark-induced etiolation. In addition to their normal cellular functions, these are now considered as a major group of stress related proteins, which function through cross-talk with other stress related proteins to decrease cellular damage.

In many studies, while analyzing global changes of gene expression analysis, the expression pattern of Hsps was found to be majorly altered under almost all type of abiotic stresses like salt, cold, drought and high light (Keeler et al. 2000; Adam et al. 2001; Queitsch et al. 2000). However, evidences for the direct involvement of these proteins under abiotic stresses except heat stress are very few. A study revealed that cisgenic *Arabidopsis* plants with altered AtHsp100 protein survived as high as 45 °C (1 h) temperature stress and also showed vigorous growth after the removal of stress (Katiyar-Agarwal et al. 2003). The transgenic rice lines over expressed with AtHsp101 showed re-growth in the post-high temperature stress recovery phase while the untransformed plants could not recover to the similar extents (Spiess et al. 2004). The updated list of the transgenic plants developed for Hsp100 is given in the Table 14.1.

14.7 Chaperonins

Molecular chaperonins are a part of cellular machinery that assists folding of newly synthesized proteins to their native state. Chaperonins are unique, high molecular weight cylindrical complexes which aid protein folding that is unmanageable by simpler chaperon systems (Hemmingsen et al. 1988). The term chaperonin was first suggested (Ranson et al. 1998) to describe proteins that are evolutionarily homologous to *E. coli* GroEL, a class of molecular chaperones found in prokaryotes and in the mitochondria and plastids of eukaryotes (Hartl 1996). Major examples of chaperonins include the prokaryotic GroEL and the eukaryotic equivalent Hsp60. Chaperonins are classified into two subfamilies, the GroE chaperonins (Group I) found in bacteria, mitochondria and chloroplasts (e.g. GroE and chCpn60) and the CCT chaperonins (Group II), found in Archaea and in the cytosol of eukaryotes (e.g. trigger factor 55, thermosomes and the TCP-1 ring complex) (Schroda 2004). Group I Cpn60 (also known as Hsp60), acts in the company of a co-chaperonin Cpn10 (Hsp10) in an ATP-dependent manner. While in bacteria, the Cpn10 is encoded by a single gene groES, in algae and plants, the plastid Cpn10 is encoded by multiple genes (Trosch et al. 2015). Although the bacterial Hsp10 is a ~10 kDa polypeptide, a ~20 kDa homologue comprising of two subunits is found in plastids. The two subunits are joined by a TDDVKD-linker sequence in head to tail fashion (Bukau and Horwich 1998). Hsp10 functions with Hsp60 as double-ring assemblies composed of back-to-back stacked rings of closely related rotationally symmetrical subunits (Kotak et al. 2007), assisting in folding, assembly and sorting of proteins.

There are Proteins with RNA chaperone activity that play important roles in cellular mechanisms (Semrad 2010). They prevent RNA from misfolding by loosening misfolded structures without ATP consumption. Oligonucleotide- or ribozyme-based assays were used to study RNA chaperone activity. Due to their functional as well as structural diversity, a common chaperoning mechanism or universal motif has not yet been identified. Although the exact mechanism is not yet understood, it is believed that disordered regions within proteins play an important role.

14.8 Heat Shock Transcription Factors (Hsfs)

Under heat stress, plant induces expression of Hsp's and other defensive genes. This happens due to the presence of conserved heat shock elements (HSEs) in the promoter region of gene, which triggers transcription of *Hsp* genes in response to heat. These *cis*-acting elements consist of the palindromic nucleotide sequence (5-AGAANNTTCT-3) that serve as recognizing as well as binding site for heat shock transcription factors or simply heat shock factors (HSFs) (Hasanuzzaman et al. 2013). As it is evident that Hsfs regulate *Hsp* genes, *Hsf* gene induction system has emerged as a powerful target for manipulating levels of Hsps through transgenic approach (Zhu et al. 2006; Zhu et al. 2009; Xin et al. 2010; Lee et al. 1995). Many researchers have opted for the transgenic approach to elucidate the function of *Hsp* and *Hsf* genes. The summary of these efforts is listed in Table 14.2. Over expression of *Arabidopsis HsfB4* resulted in altered root development and early duplication of endodermis cells, whereas impaired growth was observed in rice plants with suppressed HsfC1b. A group of researchers have successfully altered the expression of Hsps by making a change in the transcription factor (*AtHSF1*) responsible for activation of Hsps in *Arabidopsis* plants and able to produce heat stress tolerant

Table 14.2 Particulars on transgenic plants developed by using different classes of Hsf genes for high temperature tolerance

S. No	Gene	Source	Transgenic	Promoter	Reference
1	*Hsf1*	*A. thaliana*	*A. thaliana*	35s	Lee et al. (1995)
2	*Hsf3*	*A. thaliana*	*A. thaliana*	35s	Prandl et al. (1998)
3	*HsfA2*	*A. thaliana*	*A. thaliana*	35s	Li et al. (2005)
4	*HsfA2e*	*O. sativa*	*A. thaliana*	ZmUbi1	Yokotani et al. (2008)
5	*HsfA1*	*S. lycopersicon*	*S. lycopersicon*	35s	Mishra et al. (2002)
6	*Hsf3*	*A. thaliana*	*A. thaliana*	35s	Panchuk et al. (2002)
7	*HsfA1*	*G. max*	*G. max*	35s	Zhu et al. (2006)
8	*HsfA2*	*A. thaliana*	*A. thaliana*	35s	Ogawa et al. (2007)
9	*HsfA3*	*A. thaliana*	*A. thaliana*	35s	Yoshida et al. (2008)
10	*Hsf 7*	*O. sativa*	*A. thaliana*	35s	Liu et al. (2009)
11	*Hsf 1*	*B. hygrometrica*	*A. thaliana, N. tabacum*	35s	Zhu et al. (2009)
12	*HsfA2*	*L. longiflorum*	*A. thaliana*	35s	Xin et al. (2010)
13	*HsfC1b*	*O. sativa*	*O. Sativa*	ZmUbi1	Schmidt et al. (2012)
14	*Hsf A1a*	*A. thaliana*	*A. thaliana*	35s	Qian et al. (2014)
15	*Hsf A3*	*T. aestivum*	*A. thaliana*	35s	Zhang et al. (2013)
16	*Hsf A3*	*S. lycopersicon*	*A. thaliana*	35s	Li et al. (2013)
17	*HsfA1*	*L.longiflorum*	*A. thaliana*	35s	Gong et al. (2014)
18	*HsfA6f*	*T. aestivum*	*T. aestivum*	HVA1s	Xue et al. (2015)
19	*HsfA1d*	*T. salsuginea*	*A. thaliana*	35s	Higashi et al. (2013)
20	*HsfA2d*	*T. aestivum*	*A. thaliana*	35s	Chauhan et al. (2013)

Arabidopsis (Prandl et al. 1998). Over expression of *Athsf3* in *A. thaliana* using CaMV35 promoter showed a clearly enhanced thermotolerance in transgenic plants (Panchuk et al. 2002; Mishra et al. 2002). A study revealed that over expressed tomato *HsfA1* gene showed increased thermotolerance while transgenic lines in which transgene was silenced due to co-suppression were thermosensitive (Li et al. 2005). The *Glycine max* transgenics developed by over expressing *HsfA1* showed enhanced heat tolerance through activation of Hsp70 (Zhu et al. 2009). Constitutive expression of *HsfA2* in *A. thaliana* conferred enhanced basal and acquired thermotolerance (Yoshida et al. 2008; Zhu et al. 2006). The over expression of *AtHsfA3* in *A. thaliana* caused induction of a large number of heat stress associated genes that showed enhanced heat stress tolerance (Liu et al. 2009). Over expression of *OsHsp7* in *Arabidopsis* exhibited enhanced expression of certain Hsf target genes, concomitant to increased basal heat tolerance (Zhang et al. 2013). Hsf1 from resurrection plant *Boea hygrometrica* over expressed in *A. thaliana* and *N. tabacum* showed enhanced basal and acquired heat tolerance via regulation of genes involved in stress protection and mitotic cell cycle (Zhu et al. 2009). The over expression of *hsfA2* from *L. longiflorum* in *A. thaliana* activated *Hsp101*, *Hsp70*, *Hsp25.3* and *APX2* genes, resulting into heat tolerance of the transgenic plants (Lee et al. 1995). Transgenic *A. thaliana* over expressing wheat *HsfA3* showed increased thermotolerance (Li et al. 2013). Over expression of tomato *HsfA3* in *Arabidopsis* showed increased levels of several Hsp transcripts and increased heat tolerance (Higashi et al. 2013). Transgenic *A. thaliana* plants over expressing *HsfA1d* from *Thelluginella salsuginea* developed enhanced thermotolerance via induction of *AtHsfA1* regulon in the transgenic plants (Chauhan et al. 2013). Over expression of *TaHsfA2d*, which is expressed mainly in developing seeds, conferred higher tolerance to heat, salinity and drought stresses in *A. thaliana* in terms of higher survival rate, yield and biomass accumulation (Gong et al. 2014). Increased heat resistance was noted in transgenic *A. thaliana* plants over expressing a novel class of *AtHsfA1*, *LlHsfA1* from *L. longiflorum*, which was found to interact with *LlHsfA2* (Xue et al. 2014). Wheat plant over expressing *TaHsfA6f* showed tolerance to high temperature (Sakuma et al. 2006a). The updated list of the transgenic plants developed for Hsfs are summarized in the Table 14.2.

14.9 Heat Shock Promoters

During the last decade, several candidate genes, pathways and strategies have been identified by various groups across the globe and provided insight in plant heat stress adaptation. Nevertheless, we are still far from complete understanding of the molecular basis and regulatory mechanisms of abiotic stress adaptations, especially in crop plants. The regulated expression of transgenes in plants has attracted as one of the best approach in minimizing stress damage. Strong constitutive promoters are routinely used in plant transformation with a regulated expression of stress-responsive genes resulting in serious penalties on plant development with overall negative performance of transgenics. The use of stress inducible promoters may be more reliable for regulated expression of stress-responsive transgene for achieving the desired stress tolerance. Serious shortcomings on plant growth and development with overall negative performance of transgenics were observed when constitutive promoter was used for generation of transgenics (Sakuma et al. 2006b; Augustine et al. 2015b). Still, most of the researchers follow CaMV35S based expression for generation of stress tolerant transgenic plants (Table 14.3). Only few examples are available where investigators have examined alternative promoters like ubiquitin (Matsuura et al. 2013; Glover and Lindquist 1998). Since constitutive promoters are hampering the final productivity, it is important for us to identify and isolate heat-stress-inducible promoters and use them while developing transgenic crops. A typical *Hsp* gene is tightly regulated and rapidly and transiently activated upon stress. This happens as heat shock elements present in the promoter region of the *Hsp* genes, that makes Hsp promoter an ideal candidate for heat stress responsive promoter for generation of transgenic plants (Khurana et al. 2013). However, only few examples are available on the use of Hsp promoters for the transcriptional regulation of stress-related genes. The use of stress-related genes under transcriptional control of inducible promoters may minimize the adverse effect of the exogenous gene at phenotypic level. A prevailing approach for quantifying the activity of any

Table 14.3 Genetically modified plants advanced with diverse classes of Hsp promoters

S. no	Promoter	Source	Transgenic	Reference
1	Hsp18.2	*A. thaliana*	*A. thaliana*	Takahashi et al. (1992)
2	Hsp81	*A. thaliana*	*A. thaliana*	Yabe et al. (1994)
3	Hsp18.2	*A. thaliana*	*N. plumbaginifolia*	Moriwaki et al. (1999)
4	Hsp18.2	*A. thaliana*	*N. tabacum*	Lee et al. (2007)
5	HSP101	*O. sativa*	*O. sativa*	Proveniers and van Zanten (2013)
6	Hvhsp17	*H. vulgare*	*H. vulgare*	Freeman et al. (2011)
7	sHSP26	*T. aestivum*	*A. thaliana*	Khurana et al. (2013)

heat-shock promoter is by fusing the promoter of heat-shock gene to reporter genes such as GFP or GUS. This permits measuring the developmental and tissue-specific expression of genes with or without heat stress (Takahashi et al. 1992). There are few examples where Hsp promoters are fused with reporter or other gene. Hsp18.2 promoter fused to the *UidA* gene transgenic *Arabidopsis* plants showed that heat stress induced the *UidA* gene activity in almost all the organs of the plant (Lee et al. 2007). Similarly, *AtHsp18.2* promoter has been successfully used in *N. plumbaginifolia* (Moriwaki et al. 1999) and *N. tabacum* hairy roots (Yabe et al. 1994). Likewise, heat-shock-induced GUS activity was observed in transgenic *Arabidopsis* when the promoter of *Hsp81* gene was used (Crone et al. 2001). GmHsp17.5E promoter in all the organs and tissues of the flower is found to be differentially expressed in heat stress (Saidi et al. 2007). Moreover, the inducibility of GmHsp17.3B promoter was studied in the moss *Physcomitrella patens* (Proveniers and van Zanten 2013). This intricacy is now being divided into features like heat shock elements (HSEs), heat-shock factors (HSFs), and possible receptors of the heat-shock response, signaling components, and chromatin remodeling aspects (Wu et al. 2009). Transgenic rice seedlings expressing OsWRKY11 transcription factor under the rice HSP101 promoter were shown to survive longer and lose less water under a short, severe drought treatment, than wild type plants (Freeman et al. 2011). Transgenic wheat showed lower expression of *uidA* (beta-glucuronidase, GUS) reporter gene in older tissues, when *uidA* gene was fused with *HvHsp17* promoter but expression in other organs and tissues was normal. This observation was recorded upon induction of Hsp-GUS expressed transgenic plants (Nollen and Morimoto 2002). The deletion analysis of *Ta*Hsp26 promoter revealed the mechanism underlying *Ta*Hsp26 mediated regulation of heat tolerance. This study was done to characterize *Ta*Hsp26 promoter from wheat and *Arabidopsis* to generate transgenic plant (Takahashi et al. 1992). Although there are some reports on heat-stress inducible promoters, many gaps need to be filled to evaluate their role in crop plants. List of the transgenic plants developed for Hsp promoters is listed in the Table 14.3.

14.10 Signaling Molecules Involved in the Heat Stress Response

Acquired stress tolerance in plants is the result of various stress response mechanisms that act synergistically to bring favourable changes at physiological, biochemical and molecular level to prevent cellular damage during stress conditions. Substantial number of reports suggest that the Hsfs/Hsps interact with signalling molecules like growth hormones, protein kinases, cell cycle and cell death regulators and also with stress inducible proteins involved in redox regulation (glutathione and thioredoxin), antioxidants (ascorbate peroxidase) and osmolytes (trehalose, glycine-betaine and proline), and defense responses (Wang et al. 2014; Driedonks et al. 2015; Reddy et al. 2009; Baniwal et al. 2007). Interaction of Hsfs with other proteins determines their activity and function. For example, HsfA1 interact with HsfA2 to form super activator complex to induce expression of heat stress responsive genes. In contrary, interaction of HsfA5 with HsfA4 inhibits the activity of the HsfA4 through DNA binding (Lee et al. 2007; Fragkostefanakis et al. 2015). Members of class B Hsfs lack activation domain and therefore interaction with HsfA members is required for their function. In *Arabidopsis*, the activity of HsfA2 seems to be regulated by direct interaction of two co-chaperones, ROF1 and ROF2 with Hsp90 by either activating or repressing heat stress response respectively (Meiri et al. 2010). The regulation of Hsf activity is further complicated by interaction with non-chaperones like heat shock binding protein (HSBP). Hsfs exist as monomers and associate with Hsp70 and Hsp90 in the cytoplasm. The redox signalling moleculeH_2O_2 regulates Hsf activity through MAPK pathway during heat and oxidative stresses (Driedonks et al. 2015). Hsf interactions with ROS signalling molecules and scavenging enzymes have been well demonstrated (Jung et al. 2013). HsfA2 was found to be required for expression of H_2O_2 scavenging enzymes Apx1 and Apx2. In *Arabidopsis*, HsfA4a regulates expression of Apx1 through Zat12 transcription factor.

Our earlier work revealed the presence of Hsf binding *cis*-elements in the promoter region of PgApx, suggesting the interaction with ROS scavenging enzymes during heat stress. Apart from heat and oxidative stress, Hsfs involved in several stress responses including salinity and anoxia. The role of Hsf in calcium signalling is through interaction with both $Ca^{2+/}$calmodulin (CaM) and protein phosphatase (PP7). The mechanism by which CaM regulates Hsf is through interaction and phosphorylation of HsfA1a by CaM-binding protein kinase 3 (CBK3) that results in activation and binding of Hsf to HSE present in Hsp promoters (Liu et al. 2008). Wang et al. (2016) identified and validated 430 interactors of Hsp70 through co-localization and function based method in rice. Hsp90 associate with multichaperone complexes with Hsp70 and various co-chaperones such as HIP (Hsp70 interacting protein), HOP (Hsp70/Hsp90 organizing protein), Hsp40 and p23. The Hsp90 is regulated by different abiotic stresses and hormones indicating its role in stress tolerance networks. The plasma membrane H^+-ATPase (PM H^+-ATPase) plays an important role in signal transduction during cell expansion, intra cellular p^H

and stomata regulation during soil salinity. It has been shown that J3 chaperone (Hsp40-like) interact and repress the Salt Overly Sensitive2 (SOS2) like protein kinase5 that negatively regulates PM H^+-ATPase (Yang et al. 2010). Role of Hsps not only confined to countering abiotic stresses but also in biotic stress conditions. In an effector triggered immunity, precise regulation of R proteins is important for survival of plants. Studies support that Hsp90 plays crucial role along with RAR1 and suppressor of G2 allele of skp1 (SGT1) in regulation of R proteins (Seo et al. 2008). Hsp90-associated chaperonin activity is regarded to be an important factor for pathogen-triggered immunity. Defense against rice blast fungus requires chitin receptor (Cerk1) that transport from endoplasmic reticulum to the plasma membrane, which requires formation of Hsp90-HOP complex (Chen et al. 2010). In addition to Hsp90, Hsp70 is also important for defence response. From the available data, it is clear that plant immunity and heat response are connected through involvement of Hsfs and Hsps in defense response. The transition from vegetative to reproductive development in plants is controlled by multiple flowering pathways, which converge at the integrators, Flowering Locus T (FT) and Suppressor of over expression of Constans1 (SOC1). Expressions of these integrators are suppressed by flowering regulator Short Vegetative Phase (SVP). DNAJ HOMOLOG 3 (J3) of *Arabidopsis* expression is regulated by multiple flowering pathways and loss of function results in late flowering. It has been shown that J3 interacts directly with SVP and prevents binding of SVP to regulatory elements of SOC1 and FT there by promotes floral transition (Shen et al. 2011). During gametophyte development, abundant presence of Hsfs and Hsps supports the role of these proteins in floral development. Apart from this, sHsp's are also involved in early embryogenesis as evident in *Arabidopsis*, where double mutant for sHsps leads to seed abortion (Dafny-Yelin et al. 2008). Above evidence supports the role of Hsf/Hsp network in different plant developmental processes.

14.11 Genomic Approaches for Heat Stress Tolerance

DNA based molecular markers developed through contemporary technologies have become indispensable tools of plant breeding in enhancing genetic gains. Most of the studies on Hsps in relation to heat stress tolerance were either based on isolation and characterization of genes or *in vivo* expression analysis experiments but less attention has been paid towards marker assisted breeding compared to other abiotic traits like drought, salinity and cold. This could be due to the less availability of genetic resources and more complex nature of the trait. Linkage analysis based genetic mapping is the classical approach to identify QTLs related to quantitative traits. Mohammad et al. (2008) identified 3-heat stress tolerant QTLs in wheat RIL population based on stress susceptibility index (SSI) that explain 44.3 %, 27.3 % and 16.7 % phenotypic variance susceptibility. Apart from the markers associated with above QTLs. Yang et al. (2002) identified two more markers that could detect same QTLs but with additive effect for heat tolerance. In another independent study, five QTLs responsible for pollen stability at high temperature were identified in maize RIL population (Frova and Gorla 1993). But the recent revolutions in sequence technologies offered new genomic tools by which complex traits can be dissected and targeted more accurately and efficiently compared to SSR markers. In an independent study two QTLs related to heat tolerance were mapped in rice on chromosomes 3 and 4 using SSR markers (Lang et al. 2015). Using these markers, Lang et al. (2015) could successfully select homozygous plants through MABC program and this stood as successful example of molecular breeding. Hsp exhibit high genetic diversity that makes plants to behave differentially under heat stress. These allelic variations from natural populations can be captured using SNP markers and can be diploid in selection of superior genotypes in breeding programs. Identifying the naturally occurring allelic variations, that are functionally different from wild type and those that influence the target traits is really challenging. Using Eco-TILLING technology 11 SNP were identified in barley Hsp17.8 and their functional relevance to heat tolerance was evaluated. Garg et al. (2012) could identify a significant SNP that can change function of Hsp16.9 in wheat and successfully converted into breeder friendly marker. Ye et al. (2015) identified six-heat tolerance QTLs at flowering stage from two rice bi-parental populations using 6K SNP chip. Among these, two QTLs (q*HTS*1.2 & q*HTSF*6.1) contain Hsp genes, and this explains the role of Hsps in pollen fertility during heat stress in rice. This is supported by another independent study where Hsp101 was mapped on QTL region, identified for heat stress tolerance in *Arabidopsis* (Thudi et al. 2014).

Next Generation Sequencing (NGS) techniques can aid in the sequencing of condition, stage and tissue-specific transcriptome identification of heat, drought stress responsive genes, and helps in development of robust stress-associated molecular markers and construction of genetic and physical maps. This will help to elucidate key genes and metabolic pathways affected by heat and drought stresses, increase the adoptiveness and accuracy of breeding practices and accelerate crop improvement through genomics-assisted breeding. Thudi et al. (2014) identified significant SNPs associated with heat tolerance in chickpea using GBS based genome wide association studies and found few SNPs that fall in Hsp genes. Markers developed from these SNPs can be applied to select donors from germplasam for developing improved varieties through molecular breeding practices. But contrary results were obtained in GWAS for heat stress during flowering stage in *Arabidopsis* where no Hsps detected in genomic regions identified for heat stress tolerance. Only two Hsps were identified within 20 kb of moderately associated SNPs (threshold –log (P) = 4), suggesting that allelic variation in Hsps or Hsfs is not the main cause of natural variation in heat tolerance during flowering. Bulk segregation based sequence approach is another novel NGS method through which complex traits can be dissected in much simple way than map based studies. Epigenetic studies are required to detect genetic elements influenced by environmental factor (GXE) as heat stress response differs under different agro ecologies. The available whole genome sequence information and vast genetic data of crops like maize, rice can be exploited to use in less explored/orphan crops to identify the functional polymorphism in heat tolerant genes/QTLs. Studies of molecular genetic diversity among cultivars, wild accessions and ecotypes in crop species are useful for discovery of novel QTLs and alleles responsible for heat tolerance which can be further exploited in the programmes of thermotolerance improvement.

14.12 Conclusion

Understanding abiotic stress adaptations in plants is considered more challenging owing to polygenic nature of the trait and occurrence. Heat stress, being the major component of this complexity drags attention of researchers since long. Important molecules underlying heat stress tolerance identified are Hsps and Hsfs, showing chaperonin activity on various proteins of importance. Classification of different Hsps and the metabolic pathways involved are summarized to the best understanding. Role of Hsps and Hsfs as functional candidates in heat stress tolerance and other developmental pathways has been discussed with case studies. Though structural and functional characterization of Hsps/Hsfs established, their wide applicability in crop plants is still lagging due to unavailability of genetic and genomic resources. The recent revolutions in the field of genomics together with phenomics, offer exiting molecular tools which can be employed to breed heat tolerant crops. Further the cross talk molecules underlying heat stress tolerance during complex abiotic stress conditions need to be dissected.

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