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Structural and Functional Diversity of Plant Heat Shock Factors

Vivek Raxwal[1] • Surekha Katiyar-Agarwal[2] • Manu Agarwal[1*]

[1] Department of Botany, University of Delhi, Delhi 110007, India
[2] Department of Plant Molecular Biology, University of Delhi, New Delhi 1100021, India
Corresponding author: * **agarwalm71@gmail.com**

ABSTRACT

Eukaryotic response towards abiotic and biotic stress is mediated by production of molecular chaperons like heat shock proteins (HSPs), which protect cellular proteins from damage. These HSPs are under tight regulation of transcription factors known as Heat Shock Factors (HSFs). They bind to the palindromic repeat motif, Heat Shock Element (HSE), present in promoter of stress responsive genes and modulate their expression. Plants have multi member HSF family as compared to other eukaryotes. HSFs have conserved domains of specialised functions, which have been characterised as DNA binding domain, oligomerization domain, nuclear localisation and export signal and C-terminal activation domain. Based on structural peculiarities, plant HSFs have been grouped in three different classes: Class A, B and C. Although plant HSFs are structurally conserved family of DNA binding proteins, they are functionally diverse. Functional diversity and redundancy within HSF members has evolutionary significance in combating variety of stress conditions, which usually occurs in combinations during plant life cycle. HSFs play significant role not only in stress tolerance but also in various aspects of plant development.

Keywords: abiotic stress, heat stress, heat shock transcription factor, heat shock proteins
Abbreviations: HT, high temperature; **HSF**, heat shock factor; **HSP**, heat shock protein; **HSR**, heat shock response; **HSE**, heat shock element; **TF**, transcription factor

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INTRODUCTION

Abiotic stress limits crop productivity and distribution. The major abiotic stresses affecting the crop yield include sub- and supra-optimal temperature, high salt and drought. Plant response to abiotic stresses is manifested at different levels, and the molecular response is apparent due to accumulation of proteins commonly known as stress associated proteins. Up-regulation and accumulation of these stress proteins is because of a ubiquitous signal transduction pathway, which involves signal perception, followed by transduction of these signals by second messengers, subsequently resulting in activation of transcription factors that bind to the promoters of the stress associated genes, thereby modulating their expression.

One of the major stresses that affect cellular function is high temperature (HT). The most discernible cellular response (known as heat shock response i.e. HSR) to this fluctuation in temperature is the temporary shutdown of a bulk of activities that are non-essential for cell survival, accompanied by enormous synthesis of heat shock proteins (HSPs). The key function of these proteins is to protect cells during the ensuing stress and later help them recover from the residual aftermath of high temperatures. HSP synthesis is itself under the control of a transcription factor (TF) family of proteins known as Heat Shock Factors (HSFs; Nover *et al*. 1996; Morimoto 1998; Scharf *et al*. 1998; Schoffl *et al*. 1998). Initially, HSFs were thought to be controlling only the HSR; however, as it later turned out, they are involved in diverse biological phenomenon. They recognize and bind to the *cis*-elements known as heat stress elements (HSEs; 5′-AGAAnnTTCT-3′) present in promoter region of many genes and transactivate their expression.

The characterization of first HSF gene was pioneered by two different groups who isolated *S. cerevisiae* HSF (Sorger and Pelham 1988; Wiederrecht *et al*. 1988), which was followed by cloning homologues from other organisms (Nover *et al*. 2001). Subsequent investigations revealed that in contrast to a limited number of HSFs present in yeast, *Drosophila* and other vertebrates, the HSF family in plants

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Invited Review

Table 1 Overview of HSE types.

Type	Sequence
Perfect-HSE	n**TTC**nn**GAA**nn**TTC**n
4P type	n**TTC**nn**GAA**nn**TTC**nn**GAA**n
3P type	n**TTC**nn**GAA**nn**TTC**n
Gap type-1	n**TTC**nn**GAA**n(5-bp)n**GAA**n
Gap type-2	n**TTC**n(1-bp)n**GAA**n(5bp)n**GAA**n
Gap type-3	n**TTC**n(2-bp)n**GAA**n(5bp)n**GAA**n
TTC-rich 1	n**TTC**n(1bp)n**TTC**n(6-bp)n**TTC**n
TTC-rich 2	n**TTC**n(5bp)n**TTC**n(4-bp)n**TTC**n
TTC-rich 3	n**TTC**nn**TTC**n(8-bp)n**TTC**n(1bp)n**TTC**n
TTC-rich 4	n**TTC**n(3bp)n**TTC**n

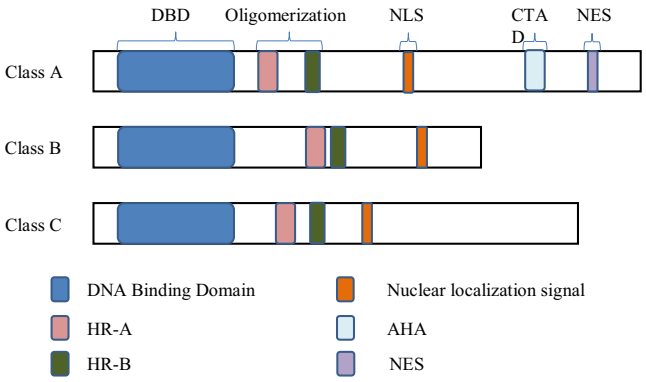

Fig. 1 Basic structure of HSFs. The DNA binding domain (DBD; blue box) is at the N-terminus, followed by the oligomerization domain that consists of Heptad Repeat A (HR-A; pink box) and B (HR-B; green box). Nuclear Localization Signal (NLS; red box) and C-Terminal Activation Domain (CTAD; light blue box) and Nuclear Export Signal (NES; violet box) are at the C-terminal portions. Class B and C do not have a CTAD.

is substantially larger (Nover *et al*. 2001; Baniwal *et al*. 2004). More than 250 HSFs have been discovered from 9 different plant species (Scharf *et al*. 2012). It is plausible, that many of these act redundantly; however, as it will be discussed in the ensuing sections, HSFs in plants have diversified to carry out specialized functions. The aim of this review is to provide an overview of structural and more importantly functional diversity of HSFs in plants.

STRUCTURE AND CLASSIFICATION OF HSFs

The basic structure of HSFs is conserved across kingdoms and is largely modular. The framework of HSFs consists of an N-terminal DNA Binding Domain (DBD), an adjacent Oligomerization Domain (OD), Nuclear Localisation Signal (NLS), C-terminal Activation Domain (CTAD) and a Nuclear Export Signal (NES; Nover *et al*. 1996; Lyck *et al*. 1997; Peteranderl *et al*. 1999; Kotak *et al*. 2004). A generalised structure of HSFs is presented in **Fig. 1**.

The DNA binding domain appears to be analogous to helix-turn-helix family of proteins and consists of 3 helical bundle packs against a small 4 stranded antiparallel β-sheets as revealed by NMR solution and crystal structure of mammalian as well as plant HSFs. The binding of HSF with HSE is mediated by the second and third helix-turn-helix motif. HSFs binding specificity to HSE is governed by the hydrophobic region of DNA binding domain (Damberger *et al*. 1994; Harrison *et al*. 1994; Vuister *et al*. 1994; Schultheiss *et al*. 1996). Binding of HSFs to HSEs is a prerequisite for the activation of heat stress and other genes harbouring HSEs. The canonical HSE consists of palindromic sequences (5′-AGAAnnTTCT-3′), the number of which should be greater than two in the promoter region for HSF (Nishizawa-Yokoi *et al*. 2011). There are multiple variations of HSEs that can present a binding site for HSFs. The sequence of canonical HSEs and their variants are presented in **Table 1**.

In-vitro binding studies with plant HSFs indicate that they bind strongly to 3P and 4P type of HSEs (Guo *et al*. 2008; Enoki and Sakurai 2011; Mittal *et al*. 2011). However, binding of plant HSFs with other variant types of HSEs has also been observed (Guo *et al*. 2008; Enoki and Sakurai 2011; Mittal *et al*. 2011). Moreover, members of rice HSF family have differential binding abilities with respect to both the perfect and variant HSEs, indicating that these HSFs might be involved in regulating diverse set of genes *in vivo* (Mittal *et al*. 2011). In addition to HSEs, HSFs can also bind to other elements known as stress responsive element (STRE; Guo *et al*. 2008). Plants HSFs consist of single intron present immediately upstream of helix-turn-helix motif thereby dividing DNA binding domain in two parts. Though the intron size is variable, its position is conserved in Arabidopsis (Nover *et al*. 2001).

Connected to the DBD with the help of a flexible linker is the oligomerisation domain also known as HR-A/B region (for Heptad Repeat A/B). The HR-A/B region derives its name from the reoccurrence of a hydrophobic amino acid at seventh position repeatedly (Nover *et al*. 2001). The HR-A and -B regions are discontinuous in case of A and C class of HSFs whereas they form a contiguous stretch in B class members. In A class members and C class members, 21 and 7 amino acids (aa) separates HR-A from HR-B, respectively.

Adjacent to HR-A/B at their C-terminus is a cluster of basic amino acids that determines the nuclear localization of HSFs. The nuclear localization of HSFs is determined by balance between NLS (Nuclear Localization Signal) and a hydrophobic lysine-rich motif (also known as NES) at the C-terminal portion of HSFs (Scharf *et al*. 1998; Heerklotz *et al*. 2001). Deletion or mutation in NES results in exclusive localization of A-classs HSFs in nucleus in Arabidopsis (Kotak *et al*. 2004). The CTAD of class A-HSFs is located between the NLS and NES and is characterized by presence of AHA motifs consisting of Aromatic (W, F, Y), large Hydrophobic (L, I, V) and Acidic (E, D) amino acids. These AHA motifs are largely responsible for transcriptional activity associated with A-class members (Nover *et al*. 2001; Scharf *et al*. 2012).

HSFs in plants have been classified in three different groups based on the differences in the basic structure of their modules. For example, B and C- group members do not have a CTAD or AHA motifs and the oligomerization domain in B-class is compact whereas A and C-class have linkers separating the HR-A and -B regions (Nover *et al*. 2001). Further sub-classification in HSF has been done on the basis of certain signature sequences and a new web tool for identification and classification of HSF is available at www.cibiv.at/services/hsf (Scharf *et al*. 2012).

FUNCTIONAL DIVERSITY OF PLANT HSFs

Though there is a great deal of structural similarity amongst plant HSFs, subtle differences within the modules, account for the different classes. In-line with the structural similarity, functional redundancy in HSFs is observed in their subtypes. However, despite the redundancy in functions of plant HSFs, distinct non-overlapping functions can be assigned to individual HSFs. It is tempting to speculate that the minor differences in their structure account for their functional uniqueness. Discovery of multiple HSFs as a result of genome sequencing efforts in Arabidopsis and rice coupled with their temporal and spatial expression studies instigated interrogations for HSFs contribution in diverse biological phenomenon. In the following sections, we will discuss the functional roles of different classes of HSFs.

Role of subclass A1 members

The quest for finding a master regulator for heat shock response mediated by HSFs was accomplished by the functional studies carried on tomato HSFs. In an attempt to over-express HSFA1a, co-suppression transgenic tomato lines impaired in the expression of HSFA1a were also obtained. Tomato HSFs were amongst the initial plant HSFs

that were isolated and functionally studied (Scharf *et al*. 1990, 1993; Treuter *et al*. 1993). Tomato genome encodes for more than 15 members in HSF family, of which HSFA1 has been shown to function as master regulator of heat stress response. Over-expression of HSFA1 resulted in increased expression of HSFA2, HSFB1, HSP17-CI, HSP104 and HSP70 whereas the HS-induced accumulation of these genes/proteins was greatly reduced or abolished in the co-suppression lines. Both the over-expression and co-suppression lines had normal growth and development; however, the co-suppression lines were severely compromised in their ability to withstand high temperatures (Mishra *et al*. 2002).

Despite similarity in Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*) HSF families, initial efforts to identify a master regulator of HSR in Arabidopsis were largely unsuccessful, owing to the redundancy in Arabidopsis HSFA1 family. Preliminary insights into the functional redundancy of HSFA1 members were obtained by studying the HSR of either over-expression lines of HSFA1 members or by utilizing their knock-out (KO) T-DNA mutants. Over-expression of either HSFA1a (Lee *et al*. 1995) or HSFA1b (Prandl *et al*. 1998) resulted in constitutive expression of HSP genes and concomitant increase in basal thermotolerance. However, the acquired thermotolerance was unaffected in both the cases, thereby indicating that neither AtHSFA1a nor –A1b is sufficient for both basal and acquired tolerance to high temperature stress.

Further insights into the function of AtHSFA1 members were gained when their functional knock-out mutants were studied. Molecular analysis of *hsfa1a/hsfa1b* single and double mutants shows that loss of either HSFA1a or HSFA1b had no obvious effects on the HSR. However, double mutant of *hsfa1a/hsfa1b* were incapable of forming HS-dependent HSE-binding complex thereby resulting in lower expression of HSF target genes (HSP18, HSP 17.6, HSP 83.1, HSP 70 and HSP101) during the early phase of high temperature stress. Additionally, expression of two of the heat inducible B-class HSFs i.e., HSFB1 (HSF4) and HSFB2b (HSF7) was reduced in the *hsfa1a/hsfa1b* double mutant. The B-class HSFs have been shown to function both as a co-activator (along with HSFA1a) and a repressor (Bharti *et al*. 2004; Ikeda *et al*. 2011). It is likely that HSFB1 and B2b function as modulators during the late phase of HSR as their reduced expression in this phase can be directly correlated with that of their target HSP genes. Though, the *hsfa1a/hsfa1b* double mutants exhibited reduced levels of HSPs, phenotypically it showed similar levels of basal and acquired tolerance to high temperature stress as the WT plants. However, electrolyte leakage assays showed that the double mutant was more sensitive to heat stress than the WT plants (Lohmann *et al*. 2004). Inability of the double mutant to exhibit a visible heat sensitive phenotype, signify that unlike tomato, the HSR in Arabidopsis is not controlled by a solitary HSF. The subtle thermo-sensitive nature of the double mutant indicates that there is partial loss of HSR in these plants. To elucidate the molecular response of the double mutant, microarray studies were carried out (Busch *et al*. 2005). Detailed analysis of this study showed that expression of a majority of LMW HSPs, three of the HS-inducible HSFs (HSFB1, -B2a and A7a) and raffinose biosynthesis pathway mediated by *GolS1* gene is regulated by AtHSFA1a and AtHSFA1b. The involvement of AtHSFA1a in the induction of *GolS1* is obscure as over-expression of AtHSF1b alone is sufficient for constitutive accumulation of the otherwise HS-inducible *GolS1* transcript (Panikulangara *et al*. 2004). Moreover, HSFA1b protein can physically bind to the promoter of *GolS1 in vitro* (Panikulangara *et al*. 2004). However, it is possible that both AtHSFA1a and -A1b are required for induction of raffinose biosynthetic pathway genes upstream to *GolS1* (Busch *et al*. 2005).

HSFA2 is a major player of the HSR in crop plants (see section on HSFA2). The expression of HSFA2 in tomato is apparently controlled by HSFA1a. To determine whether similar regulation exists in Arabidopsis, chimeric repressor silencing technology was used to functionally repress the activator function of A-class HSFs. Constitutive over-expression of dominant negative forms of either HSFA1d or HSFA1e, resulted in reduced accumulation of HSFA2 under high light as well as high temperature. These results were substantiated by reduced expression of HSFA2 in independent knock-out mutants of *hsfa1d* and *-a1e* indicating that both these HSFs are involved in regulation of HSFA2 expression (Nishizawa-Yokoi *et al*. 2011). However, the HL-induced expression of AtHSFA2 was not abolished in *hsfa1d/hsfa1e* double KO mutants, indicating that unlike tomato HSFA2, whose expression is completely lost in the pericarp of HSFA1a co-suppression lines (Mishra *et al*. 2002), expression of AtHSFA2 is dependent on HSFA1d, HSFA1e and some other transcription factors. It was further observed that the cosuppression lines in tomato accumulated high levels of HSFA1a siRNAs and these small RNAs could be responsible for targeting other HSFs resulting in loss of their function as well. Therefore, like Arabidopsis, reduction or disappearance of HSFA2 in tomato cannot be attributed solely to the functional loss of a single HSF. HSFA1d/A1e also induces expression of HSFA7a,-A7b, -B1 and -B2a under both HL and HS conditions, indicating expression of HSFA7a, -B1 and -B2a is redundantly controlled by HSFA1a/A1b and HSFA1d/A1e pairs. The decreased expression of HSPs in the *hsfa1d/hsfa1e* KO mutant correlated with its reduced ability to acquire thermotolerance (Nishizawa-Yokoi *et al*. 2011).

Both the *hsfa1a/a1b* and *hsfa1d/a1e* double mutants in Arabidopsis were not significantly compromised in their ability to withstand high temperatures, thereby facilitating further investigations for identification of heat stress master regulator. A series of triple and quadruple KO (QKO) mutants were generated to decipher the HSR pathways controlled by *Arabidopsis thaliana* A-class HSFs (Liu *et al*. 2011). Though the QKO mutants harboured defects at early stages of development, they were highly susceptible to high temperature stress at all the tested stages. Out of the different combinations of triple mutants, loss of HSFA1a,-A1b and A1d was the most critical in determining the sensitiveness of the plants to high temperature stress. Molecular analysis of the HSR genes in the triple and QKO mutants showed that HSFA1a, -A1b and A1d act redundantly in controlling the major part of HSR in Arabidopsis (Liu *et al*. 2011). The involvement of HSF-A class in mediating molecular response to osmotic, salt and oxidative stress was exemplified by sensitiveness of the QKO to these conditions. Since triple mutants were not included in phenotyping for these stresses, the hierarchal roles of individual class A HSF members cannot be commented upon.

Role of subclass A2

HSFA2 class have emerged as one of the most important class controlling HSR in plants. The primary evidence that point to the critical role played by HSFA2 is its very strong inducibility by high temperature stress in plants (Scharf *et al*. 1998; Port *et al*.2004; Schramm *et al*. 2006; Charng *et al*. 2008; Chan-Schaminet *et al*.2009). Additionally, the inducible expression of HSFA2 is dependent on the presence of HSFA1 class members, which themselves act as master regulators of HSR (Scharf *et al* 1998; Mishra *et al*. 2002; Nishizawa-Yokoi *et al*. 2011). Ectopic expression of tomato HSFA2 rescues the thermosensitivity of HSFA1a co-suppression lines (Mishra *et al*. 2002) indicating: a) HSFA1a action is mediated through HSF2 and b) HSFA2 is a later component in plant responses to high temperature stress than HSFA1.

Small portions of C-terminal activation domains (CTAD) of tomato HSFA2 and HSFA1 can be functionally swapped (Doring *et al*. 2000) thereby further corroborating that both HSFA1 and HSFA2 manage similar pathways of HSR in plants. In addition to controlling HSFA2 expression, HSFA1a also controls its intracellular distribution (Scharf *et*

al. 1998). The majority of tomato HSFA2 is localized to cytoplasm under both control and HS conditions. However, a minor deletion of 8 or 28 amino acids from its C-terminal enables its predominant localization in nucleus, indicating that a motif in the C-terminal determines the nucleo-cytoplasmic localization of HSFA2 (Lyck *et al*. 1997). The deleted portion actually contains a NES, which is highly efficient in nuclear export of HSFA2 and hence its deletion causes nuclear retention of HSFA2 (Heerklotz *et al*. 2001). Additionally, HSFA1 mediates the nuclear import of HSFA2 via interaction with C-terminal half of Class A specific HR-A/B region (Scharf *et al*. 1998). In fact interaction of HSFA1 and HSFA2, results in formation of a hetero-oligomeric superactivator complex that synergistically activate expression of HS genes (Chan-Schaminet *et al*. 2009).

In addition to high temperature stress, the Arabidopsis HSFA2 is inducible by oxidative stress (Nishizawa *et al*. 2006; Miller and Mittler 2006). To determine the potential target genes of HSFA2, Arabidopsis T-DNA insertion mutants were exploited. Such an analysis revealed that transcripts of a large number of HSP genes show reduced expression in *hsfa2* mutant. Additionally, Ascorbate peroxidase 2 (Apx2) gene was strongly down regulated due to absence of HSFA2 (Schramm *et al*. 2006). Apx protein has been shown to scavenge intracellular reactive oxygen species (ROS) and therefore down-regulation of Apx2 in *hsfa2* mutants is a strong indication that HSFA2 plays an important role in regulating oxidative stress. These results were further substantiated by increased ROS accumulation leading to early cell death in protoplasts of *hsfa2* mutant plants (Zhang *et al*. 2009).

There is a considerable overlap between the transcripts induced by anoxia and high temperature stress in Arabidopsis as both these stresses result in quick accumulation of H_2O_2 (Banti *et al*. 2010). One of the key components induced by both these stresses turns out to be HSFA2 and its downstream targets (Banti *et al*. 2010). The acclimation to anoxia can be induced by a brief period of high temperature. However, *hsfa2* or *hsfa1a/1b* double mutants do not display this cross-acclimation ability, thereby confirming a direct role of HSFA2 in anoxia tolerance. In agreement to these results, ectopically overexpressing HSFA2 transgenic lines showed enhanced tolerance to anoxia and submergence tolerance (Banti *et al*. 2010). The tolerance of Arabidopsis plants over-expressing homologous or heterologous HSFA2 to high salt/osmotic stress (Ogawa *et al*. 2007; Yokotani *et al*. 2008), along with the above observations point to a critical role of HSFA2 as a cross-talk between different stresses, which appear to converge at generation of ROS. The constitutive presence of HSFA2 at an early stage and its HS-inducible accumulation at a later stage of anther development signifies that HSFA2 protects pollen from detrimental high temperature stress (Giorno *et al*. 2010).

Role of class B HSFs

The class B HSF members possess DBD and OD but lack transcriptional activation potential (Czarneka-Verner *et al*. 2000; Kotak *et al*. 2004). On the contrary, AtHSFB1 suppressed the transactivation of HSE:GUS mediated by HSF4a (Czarneka-Verner *et al*. 2000). The repressor domain of B-class HSFs was mapped to the N-terminal portion of its C-terminal regulatory region (CTR) and comprised of 36 amino acids (Czarneka-Verner *et al*. 2004). The role of HSF B-class as transcriptional repressors has also being confirmed by use of T-DNA mutants of Arabidopsis wherein loss-of-function of HSFB1 and HSFB2b resulted in enhanced expression of defensin genes Pdf1.2a/b. and proteins extracted from heat stressed double mutant plants formed a complex with HSE earlier than WT plants, thereby suggesting that loss of HSFB1/HSFB2b can result in early activation of HS-induced genes (Kumar *et al*. 2009).

Detailed analysis with the over-expression lines of WT and dominant negative forms of HSFB1 demonstrated that HSFB1 act as a repressor of HS gene expression during non-heat stress and moderate heat stress conditions (Ikeda *et al*. 2011). In addition to HSFB1, over-expression of HSFB2b also repressed the expression of reporter gene driven by HSFA2 promoter. In agreement with these results, microarray studies with *hsfb1/b2b* double KO mutants showed that amongst the genes whose expression was high in the mutant, there was a preponderance of HS genes. The expression of upstream HSFs i.e., HSFA2 and HSFA7a were also higher in the double mutant under moderate heat stress conditions of 28°C. At a higher degree of temperature stress (32°C) levels of HSFA2 and –A7a decreased only after 30 minutes of heat stress in the mutant. This suggests that HSFB1 and B2b act as repressors to attenuate the HSR, during extended stress at higher temperatures (Ikeda *et al*. 2011). Microarray studies further revealed that under conditions of acquired thermotolerance, a large number of genes that are positively regulated by HSFA1a and -A1b are actually down-regulated in the mutant and hence the mutants displayed lower acquired thermotolerance than the WT plants (Ikeda *et al*. 2011).

HSF B-class members can also act as gene activators under certain conditions, which has also been observed for tomato HSFB1 (Bharti *et al*. 2004). Co-expression of HSFA1a and HSFB1 synergistically enhanced HSP promoter driven GUS activity. Accumulation of the endogenous HSP17-CI protein was also higher in presence of both HSFA1a and HSFB1 than the combined effect exerted by expression of either of them. Additionally, it was shown that HSFB1 also cooperates with general transcriptional activators to enhance expression of constitutive promoters. HSFB1 and HSFA1a interact with Arabidopsis CBP (CREB Binding Protein) like protein HAC1 (histone acetyl transferase) and form a ternary complex to enhance HSP17 promoter driven GUS activity (Bharti *et al*. 2004).

Role of HSFs in plant development

Constitutive expression of HSPs at later stages of seed development and early stages of seed germination has been a subject of study by many laboratories (Wehmeyer *et al*. 1996, 2000; Hong and Vierling 2001). Despite the presence of HSEs in the promoters of many HSPs, only specific members are expressed during seed development (Wehmeyer and Vierling 2000) indicating their stringent regulation by developmental cues. One of the plant HSF that has emerged as the positive regulator of seed specific HSPs is the dicot specific HSFA9 (Scharf *et al*. 2012). Using yeast one-hybrid approach, promoter region of *Helianthus annuus* HSP17.7G4 (HaHSP17.7G4), was used to fish out HaHSF9 (Almoguera *et al*. 2002). HaHSF9 mRNA accumulated in developing seeds and disappeared in the early phases of seed germination. The accumulation of HaHSF9 transcript was not influenced by heat stress or exogenous water deficit stress. Additionally, HaHSF9 positively regulated the expression of HaHSP17.7 G1 and –G4 promoters in transient assays (Almoguera *et al*. 2002). The embryo specific expression of HaHSF9, together with its ability to transactivate developmentally regulated promoters of HSPs, indicates a unique role of HSFA9 in seed development.

Similar to HaHSF9, the Arabidopsis homologue *AtHSFA9* transcript starts accumulating in developing seeds and its level is highest in dry seeds. Class I (HSP17.4-CI), Class II (HSP 17.7 CII) and HSP101 were identified as potential targets of HSFA9 as their expression strongly correlated with HSFA9 during seed maturation stages (Kotak *et al*. 2007). Further investigations revealed that the promoter of HSFA9 gene contains a RY/Sph motif, which presents a binding site for the seed specific transcriptional activators ABI3 and FUS3. Moreover, expression of ABI3 and FUS3 transcripts precede HSFA9 transcript accumulation, further indicating that ABI3 or FUS3 might regulate HSFA9 expression in seeds. Transient assays indeed revealed that ABI3, not FUS3, led to the accumulation of HSFA9 driven GUS and this transactivation is mediated by RY/Sph motif (Kotak *et al*. 2007). The absence of HSFA9

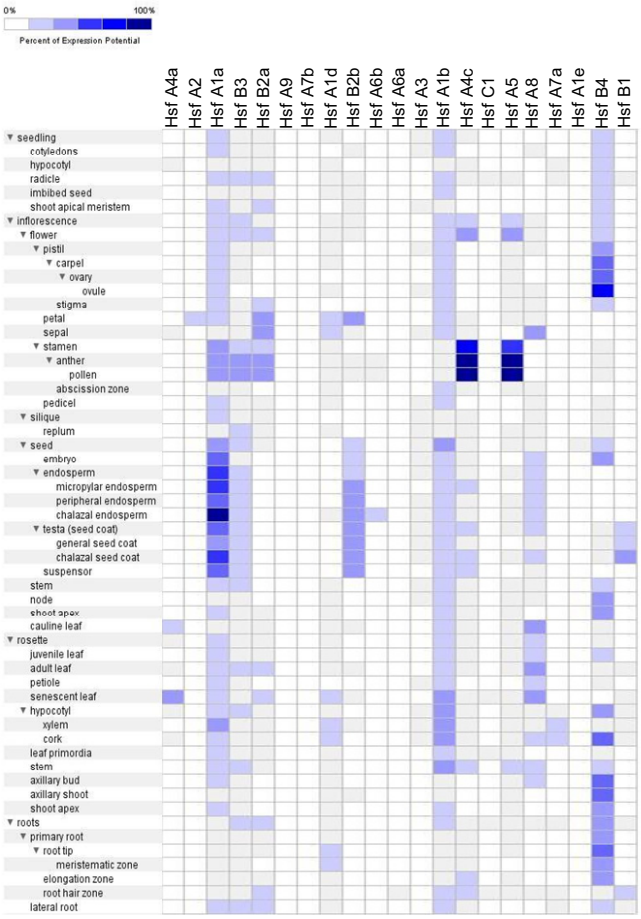

Fig. 2 Normalised and averaged signal intensities presented in the form of heat maps (see colour code) is representation of Arabidopsis HSF transcripts relative abundance in developmental stages. The data for different anatomies was extracted from AtGenExpress microarray experiments and details of the samples can be found at http://arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp

in *abi3* mutants, and its accumulation in an ABA-dependent manner in leaves of plants ectopically expressing ABI3, further point to the ABI3 dependent regulation of HSFA9 (Kotak *et al*. 2007).

The appearance of HSF9 and target HSPs during seed desiccation stages, apparently indicates, their role in cellular protection against water loss. Though AtHSF9 is regulated by stress hormone ABA via ABI3, overexpression of dominant negative form of its sunflower homologue in tobacco affected expression of sHSPs without affecting seed development and germination, thereby complicating the role of HSFA9 and seed-specific sHSPs in desiccation tolerance (Tejedor-Cano *et al*. 2010). Nevertheless, HaDREB2, an AP2-domain containing transcription factor (TF), was isolated using dehydration responsive element (DRE) of HaHSP17.7G1 promoter as bait (Diaz-Martin *et al*. 2005). HaDREB2 was found to interact and function synergistically with HaHSF9 to activate expression of HSP17.7G1 promoter, thereby indicating that HSFA9 cooperates with additional TFs for seed specific regulation of HSPs (Diaz-Martin *et al*. 2005). In line with these experiments, ectopic overexpression of HaDREB2 and HaHSFA9 together, in transgenic tobacco enhanced seed longevity (Almoguera *et al*. 2009).

There is an intricate interplay between ABA and auxins during seed development in plants. HSFA9 and HaIAA27 proteins are regulated by ABA and auxin respectively and they interact with each other to control seed development. The HaIAA27 protein interacts with HaHSFA9 *in planta* and represses the activity of HaHSFA9 in transient assay (Carranco *et al*. 2010). The interaction between HaIAA27 and HaHSFA9 was observed in immature sunflower embryos, indicating that the otherwise highly labile IAA27 protein is stabilized in developing immature embryos. In the mature embryos IAA27 protein is degraded probably because of increased auxin content and thereby alleviating the repression of HaHSFA9 (Carranco *et al*. 2010). In addition to the seed specific developmental role of HSFA9, an Arabidopsis mutant, which displayed right handed root slanting and reduced gravitropic responses was defective in HSFA4c function, thereby indicating that HSFs play a much greater role in different aspects of plants development (Fortunati *et al*. 2008). Preliminary insights into the role of other HSFs mediating developmental processes can be gained from gene expression data available from public resources (**Fig. 2**).

HSFs and plant senescence

Plant senescence is a highly orchestrated active degeneration process at cellular, tissue, or organ level leading to death. Senescence in plants is generally associated with leaf senescence, which is a complex yet highly co-ordinated process. Senescence in leaves is initiated with breakdown of chloroplasts followed by hydrolysis and mobilization of macromolecules and finally disintegration of nucleus and mitochondria (Buchanan-Wollaston *et al*. 1997, 2003, 2005). Leaf senescence limits the crop yield by impeding the growth phase and therefore, it is imperative to elucidate the molecular mechanism underlying leaf senescence.

Gene expression studies of leaf senescence revealed that senescence program is accompanied by substantial changes in the expression of a suite of specific genes, commonly referred to as Senescence-Associated Genes or SAGs (Buchanan-Wollaston *et al*. 1997). Differential gene expression is often related to the transcriptional control mechanism and it is believed that transcriptional control has an important role in senescence process (Guo and Gan 2006; Balazadeh *et al*. 2008). A sizable fraction of these differentially expressed genes during leaf senescence belong to the families of transcription factors such as NAC, WRKY, C2H2 type zinc finger, AP2/EREBP, MYB, bZIP, CCAAT binding, MADs box and HSF (Buchanan-Wollaston *et al*. 2005; Balazadeh *et al*. 2008).

Leaf senescence is also regulated by abiotic factors such as extreme temperatures, drought, salinity, nutrient limitation and oxidative stress (Nam 1997; Navabpour *et al*. 2003). This is supported by the observation that almost one-third of the senescence-related TFs were found to be responsive to a number of abiotic stresses (Lim *et al*. 2007; Balazadeh *et al*. 2008). Further, overexpression of the C-repeat/dehydration responsive element binding factor 2 (*CBF2*) gene in Arabidopsis resulted in remarkable delay in the onset of developmental leaf senescence, thereby extending the life-span of these plants (Sharabi-Schwager *et al*. 2010).

The involvement of HSFs in plant senescence stems from multiple evidences. Out of 21 HSFs, 9 HSFs exhibit more than two-fold change in expression in senescence leaf as compared to the young leaf in Arabidopsis (eFP browser: http://www.bar.utoronto.ca). Of particular interest is HSFA6a that is regulated by both abiotic stress and developmental senescence (Balazadeh *et al*. 2008). Guo *et al*. (2004) studied transcriptome of Arabidopsis leaf senescence and found that 130 transcriptional regulators, including one HSF, are differentially expressed in senescent leaves. Microarray studies of Arabidopsis leaf senescence show that AtHSFB1 was significantly upregulated in senescing leaf tissue (Buchanan-Wollaston *et al*. 2005). Role of HSFB1 during leaf senescence was investigated by employing its T-DNA insertion mutant (Breeze *et al*. 2008). Although mutant plants showed no defects in growth and development when grown under unstressed conditions, considerable differences were observed in the photosynthetic efficiency of mutant and wild type plants, especially at the later stages of development. HSFB1 mutants were found to exhibit accelerated leaf senescence and reduced tolerance to

drought stress. Gene profiling studies of *hsfb1* mutant showed that while HSFB1 and HSP70 were significantly downregulated, many SAGs such as SAG12, WRKY and peroxidase genes were upregulated (Breeze *et al*. 2008). Although the study by Breeze *et al*. (2008) highlights the role of AtHSFB1 in leaf senescence, it has raised a number of questions. It is not clear how AtHSFB1 regulates leaf senescence, what are the targets of HSFs, and whether leaf senescence pathway associated with HSFs shares some common features with abiotic stress pathway. These questions can be answered by studying the role of other members of HSF family in leaf senescence. It is believed that leaf senescence involves a number of signaling components and therefore, it is a challenge to identify components of this regulatory network and elucidate complex interactions and cross-connection that occurs during leaf senescence.

ROS and HSFs

Heat stress in addition to causing disruption of diverse cellular mechanisms for example. increased fluidity of membrane lipids and loss of membrane integrity, enzyme inactivation, inhibition of protein synthesis, protein degradation and denaturation, disruption of microtubule organisation (Semertenko *et al*. 1997; Wahid *et al*. 2007), also invariably leads to generation of ROS (Volkov *et al*. 2006), which in turn can affect the activity/inducibility of HSFs. In fact H_2O_2 has been shown to activate human and Drosophila HSF1 (Zhong *et al*. 1998; Ahn and Thiele 2003). Oligomerization or conformation change of HSFs in response to ROS has not been discovered in plants, however multiple evidences indicate that some of stress signaling pathways regulate gene expression through ROS and HSFs (see above section on HSFA2). The high degree of overlap between oxidative and heat shock response has further been shown by microarray analysis of knock-out mutants of cytosolic Apx. Depletion of Apx led to increase in the steady state levels of HSFA4a and HSFA8 transcripts. Additionally, over-expression of AtHSFA4a lacking the activation domain, precluded the expression of Zat12 and Apx transcripts in response to light stress (Davletova *et al*. 2005). Interestingly, multiple members of Apx gene family are inducible by differential HS conditions (Panchuk *et al*. 2002), raising the possibility that different Apx proteins are active under mild, acute and extended heat stress conditions. Conforming to these results was accumulation of thermostable Apx^S form at high temperatures (Panchuk *et al*. 2002). The constitutive accumulation of Apx^S and its corresponding gene Apx2, in AtHSFA1b overexpressing plants further consolidated the views that HSFs and ROS are coherently linked to each other.

Role of HSFs in other stresses

The complexity of HSFs in plants, their expression patterns (**Fig. 3**) and functional studies indicate that specific HSF members play an important role in mounting cellular response to a multitude of stresses and plant development. Microarray studies in Arabidopsis plants over-expressing a constitutively active form of DREB2A, revealed up-regulation of AtHSFA3 and many HS-inducible genes (Sakuma *et al*. 2006). DREB2A was also inducible by high temperature stress and the knock-out mutants of DREB2A had reduced accumulation of AtHSFA3 and HS-inducible genes. This indicates DREB2A has a dual function in controlling the expression of water stress and heat stress responsive gene expression (Sakuma *et al*. 2006). In compliance with the levels of HSPs, transgenic plants were more tolerant to high temperature stress, whereas the *dreb2a* mutants were sensitive. Ectopic expression of the heterologous maize DREB2A Arabidopsis also resulted in accumulation of HSFA3 and enhanced thermotolerance (Qin *et al*. 2007). DREB2A physically binds to DRE elements in the promoter of AtHSFA3 and activates its expression. Mutant lines of *hsfa3* were compromised in both basal and acquired thermotolerance and this correlated with the reduced expression of HSPs in these lines (Schramm *et al*. 2008; Yoshida *et al*. 2008). In a similar study, Arabidopsis DREB2C has also been demonstrated to transactivate HSFA3 and downstream targets (Chen *et al*. 2010). Using functional complementation of a yeast mutant defective in cadmium tolerance, a wheat clone coding for HSF4a was isolated. Overexpression of TaHSFA4a in rice plants increased Cd tolerance, whereas knock-down of endogenous rice orthologue resulted in Cd sensitivity (Shim *et al*. 2009). The target genes of either TaHSF4a or OsHSF4a were speculated to be metallothionein genes. It will be intriguing to see if promoters of these genes have functional HSEs.

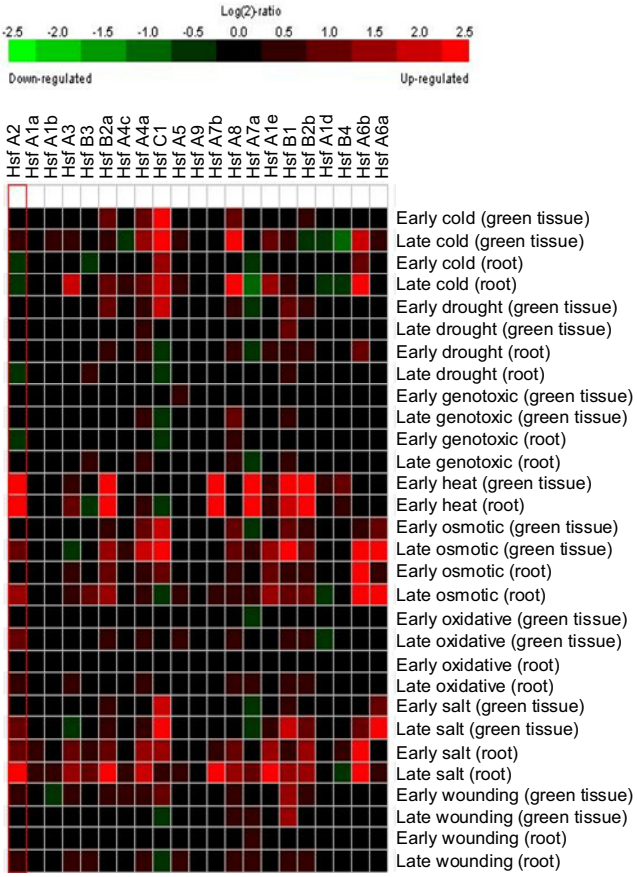

Fig. 3 Normalised and averaged signal intensities presented in the form of heat maps (see colour code) is representation of Arabidopsis HSF transcripts relative abundance in different abiotic stresses. The data for different physiologies was extracted from AtGenExpress microarray experiments and details of the samples can be found at http://arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp

CONCLUSION

A remarkable structural and functional diversity exists in plant HSFs. Their involvement in diverse biological processes indicates that plants have evolved multiple mechanisms for their survival from environmental insults. In addition to the classical HSFs, plants have a number of genes coding for HSF like proteins, whose functionality is largely unknown. The role of B-class members in acting as general gene activators as well as repressors in specific cases is intriguing and warrants further investigations. The initiation of structural and functional studies on C-class HSFs requires attention. Details on involvement of ROS signaling in HSF regulation are beginning to emerge and a complete understanding of HSF function will be useful in designing plants that can withstand multiple stresses.

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