Biochimica et Biophysica Acta xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

# The plant heat stress transcription factor (Hsf) family: Structure, function and evolution ☆

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ARTICLE INFO

*Article history:*
Received 21 July 2011
Received in revised form 6 October 2011
Accepted 7 October 2011
Available online xxxx

*Keywords:*
Heat stress
Abiotic stress response
Transcriptional regulation
Chaperone activity
Protein homeostasis
Stress tolerance

ABSTRACT

Ten years after the first overview of a complete plant Hsf family was presented for *Arabidopsis thaliana* by Nover et al. [1], we compiled data for 252 Hsfs from nine plant species (five eudicots and four monocots) with complete or almost complete genome sequences. The new data set provides interesting insights into phylogenetic relationships within the Hsf family in plants and allows the refinement of their classification into distinct groups. Numerous publications over the last decade document the diversification and functional interaction of Hsfs as well as their integration into the complex stress signaling and response networks of plants. This article is part of a Special Issue entitled: Plant gene regulation in response to abiotic stress.



## 1. Introduction: Plant stress response

The origin of terrestrial plants about 400 million years ago required special adaptations to rapidly changing environmental conditions. As sessile organisms plants had to become specialized to growth and propagation under divergent stress conditions such as low or high temperatures, high salt or heavy metal stress or extreme water deficiency. A network of interconnected cellular stress response systems is a prerequisite for plant survival and productivity challenged by global changes of climate [2–9].

Although plant stress responses were studied experimentally since the middle of the 19th century, a milestone in the analysis of cellular stress response systems was the pioneering work of F. Ritossa with the fruit fly *Drosophila*, who observed striking changes of gene activity patterns of the polytene chromosomes in larval salivary glands after heat stress (HS) [10]. The newly formed heat stress proteins (Hsps) were described by Tissieres and Mitchell [11]. As it turned out, Ritossa had discovered the central parts of a general stress response system conserved throughout the living world including all prokaryotes and eukaryotes investigated so far.

The nearly 50 years of molecular cell biology research in this field uncovered a central stress response system in cells sensing deviations of protein homeostasis, i.e. of the equilibrium between new synthesis, folding, intracellular targeting, biological function and degradation of proteins. Hs-induced and constitutively expressed members of the conserved Hsp families act as molecular chaperones. They are essential for maintenance and/or restoration of protein homeostasis [12–17]. Denaturation of proteins and problems in the processing of newly synthesized proteins during stress are assumed to result in a decrease of the pool of free chaperones. This so-called cytosolic protein stress response triggers transcription of Hsp encoding genes under the control of heat stress transcription factors (Hsfs), which are in the focus of this review. We will concentrate mainly on structure and function of plant Hsfs but will occasionally also include relevant information about Hsfs or transcription activator proteins from non-plant systems.

## 2. Modular structure of Hsfs

Similar to many other proteins regulating gene activity, Hsfs have a modular structure. Despite a considerable variability in size and sequence, their basic structure and mode of promoter recognition are conserved throughout the eukaryotic kingdom [18–20]. For the presentation in Fig. 1, we show five examples of tomato Hsfs with features typical for plant Hsfs.

☆ This article is part of a Special Issue entitled: Plant gene regulation in response to abiotic stress.

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1874-9399/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.bbagrm.2011.10.002

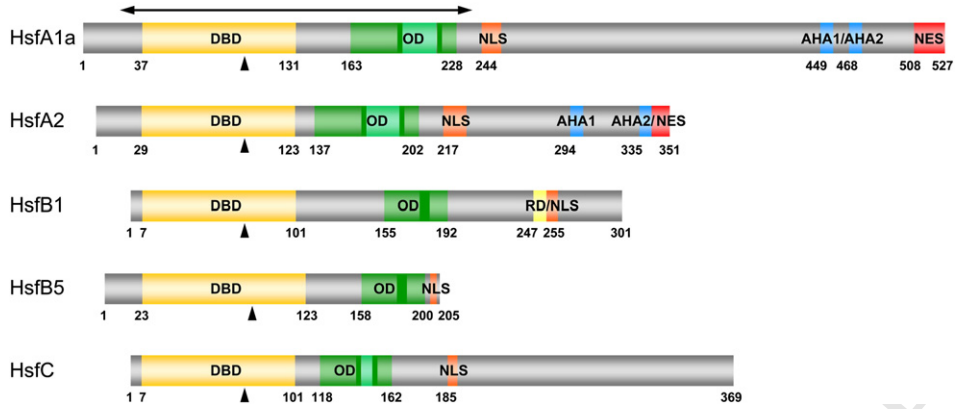


**Fig. 1.** Basic structure of Hsfs. The basic structure is exemplified for 5 tomato Hsfs, aligned at the beginning of the DNA binding domain (DBD): the position of the conserved intron is indicated by arrow heads; OD, oligomerization domain (HR-A/B region); NLS, nuclear localization signal; NES, nuclear export signal; AHA, activator motifs; RD, tetrapeptid motif as core of repressor domain. For details see Sections 2.1 to 2.6. Double headed arrow on top indicates the sequence region used for the phylogenetic analysis (Fig. 3).

### 2.1. DNA binding domain (DBD) and heat stress elements (HSE)

The highly structured DNA-binding domain (DBD) is located close to the N-terminus of all Hsfs. Crystal and NMR solution structure analyses of the DBD of selected Hsfs from *Drosophila*, yeast and plant revealed that it is formed of a three-helical bundle (H1, H2 and H3) and a four stranded antiparallel ß-sheet [21–24]. The hydrophobic core of this domain ensures the precise positioning and highly selective interaction of the central helix–turn–helix motif (H2–T–H3) with heat stress promoter elements (HSE; [25–27].

HSEs are formed of repetitive patterns of palindromic binding motifs (5′-AGAAnnTTCT-3′) upstream of the TATA box of eukaryotic HS-inducible genes [1, 28–31]). The G and C residues positioned in the major groove on opposite sites of the DNA helix are essential for HSE function [25]. Usually more than two HSE motifs are required, and in addition, details of the HSE fine structure as well as promoter or chromatin context are crucial for efficient binding of the Hsf oligomers [30, 32–35].

HSE independent binding sites for Hsfs are a matter of frequent speculations. In this context, it is remarkable that the unique Hsf in yeast is essential for survival also under non-stress conditions and that the major binding sites for the only *Drosophila* Hsf reside in non-HS genes (see Section 3.2). Concerning plants, it has to be shown experimentally whether the observed association of AtHsfA1a with so-called stress responsive elements (STRE, e.g. -AGGGG-) is relevant for Hsf-dependent expression of the corresponding genes [30]. At least, weak binding sites for Hsfs, e.g. in the promoter of house-keeping genes, may be enhanced by adjacent binding of other transcription factors as part of an enhancer complex (Section 4.4, [35]).

### 2.2. Oligomerization domain (OD)

The oligomerization domain (OD or HR-A/B region) is connected to the DNA-binding domain by a flexible linker of variable length (15–80 amino acid residues). A heptad pattern of hydrophobic amino acid residues in the HR-A/B region leads to the formation of a coiled-coil domain characteristic of leucine zipper-type protein interaction domains [36]. Based on peculiarities of their OD, we discriminate three classes of Hsfs in plants, i.e. classes A, B and C (see Fig. 1 and [1, 37, 38]). Similar to all non-plant Hsfs, e.g. yeast, nematodes, *Drosophila* and mammals [37], the HR-A/B region of plant class B Hsfs is compact, whereas class A and class C Hsfs have extended HR-A/B regions caused by insertions of 21 (class A) or 7 (class C) amino acid residues between the A and B parts (see lower case letters in the examples given below; Sl, tomato; Sc, bakers yeast; Hs, human). Interestingly, the OD of plant Hsfs confers distinct patterns of specificity for heterooligomerization (see Sections 4.3 and 4.6):

```
HR-A linker +/-insertion HR-B
SlHsfA1a:       L6aaL6aaL6aaL:RQQQqatdnqlqgmvqrlqg-
melrqQQ:MMSFLAKAV
SlHsfC1:   L6aaL6aaL6aaM:TRRLeatekrp————————QQ:
MMGFLCKVD
SlHsfB1:   L6aaL6aaL6aaA:KKQC————————————NE:
LVAFLSQYV
ScHsf1:    I6aaL6aaA6aaQ:QQAL————————————EK:
MFRFLTSIV
HsHsf1:    M6aaL6aaL6aaQ:QKVV————————————NK:
LIQFLISLV
```

### 2.3. Nuclear localization signal (NLS)

The nuclear localization signal (NLS) of Hsfs is formed by monopartite (m) or bipartite (b) clusters of basic amino acid residues C-terminal of the OD [39]. In B-type Hsfs, the basic cluster connected with the highly conserved repressor tetrapeptide motif -LFGV- (underlined, see Section 4.5) presumably serves as NLS.

```
SlHsfA1 (b): NKR5aaKKRRIK
SlHsfA4a (m): RKRRLP
SlHsfB1 (m): LFGV4aaKKKKR
SlHsfC1 (b): RSKR7aaKKRR
```

### 2.4. Nuclear export signal (NES)

Depending on the balance of nuclear import and export, the intracellular distribution of Hsfs changes dynamically between nucleus and cytoplasm [40, 41]. A hydrophobic, frequently leucine-rich nuclear export signal (NES) at the C-terminus of many Hsfs [41] is required for the receptor-mediated nuclear export in complex with the NES receptor. Together with the adjacent activator modules (AHA motifs, see Section 2.5), the NES serves as part of a type-specific signature region in the C-terminus (*) of class A Hsfs in plants ([38], see Section 2.7).

```
SlHsfA1b: AHA 37aa LKHMHNLTEQMGLL 6aa*
SlHsfA2: AHA 37aa LQDLVDQLGFL*
SlHsfA4a: AHA 35aa VISLTEQLGHL 3aa*
```

### 2.5. Activator motifs (AHA motifs)

The function of class A Hsfs as transcription activators is mediated by short activator peptide motifs (AHA motifs) located in their C-terminal domains (CTD). These motifs are characterized by aromatic (W, F, Y), large hydrophobic (L, I, V) and acidic (E, D) amino acid residues [38, 42, 43]. In Hsfs of the A3 type, the CTD does not contain distinct AHA motives but rather a characteristic pattern of tryptophane residues, which give additive contributions to the activator function [44]. Among the class A Hsfs, HsfA8-types form a marked exception since their CTDs lack any detectable AHA motif. In agreement with this, AtHsfA8 was inactive in yeast monohybrid assay and it does not recruit components of the transcription machinery in *in vitro* pull down assays [38].

SlHsfA1a: DPFWEKFLQS
SlHsfA2: DDIWEELLSE
SlHsfA4a: DVFWEQFLTE
SlHsfA3: LWG16aaLWD17aaLWD14aaKWP

Similar AHA motifs or activator regions with patterns of aromatic residues in an acidic surrounding were identified in many other transcription factors of yeast and mammals, e.g. Hsfs, VP16, RelA, Sp1, Fos, Jun, Gal4, Gcn4 (see summary and references in [38, 43]). Most likely, they represent the essential sites of contacts with subunits of the basal transcription complex. Tjian and Maniatis [45] proposed a model of cohesive interfaces, i.e. of interacting surfaces with a mutually corresponding pattern of aromatic/hydrophobic amino acid residues between activator protein and its positively charged target proteins (coactivators). In support of this concept, mutant forms with exchanges of the aromatic and/or hydrophobic residues do not interact with components of the transcription machinery *in vitro* and are deficient in reporter assays *in vivo* [38, 46–53].

### 2.6. Repressor domain of class B Hsfs

All class B Hsfs, except HsfB5, are characterized by the tetrapeptide -LFGV- in the C-terminal domain, which is assumed to function as repressor motif by interaction with a hitherto unknown corepressor in the transcription machinery [54–56]. Similar conserved -LFGV- motifs were identified as core of repressor domains in other plant transcription factors (see Section 4.5 and [55]).

### 2.7. Functional domains and signature sequences

The functional domains and motifs described before (Sections 2.1 to 2.6) are either general (NLS, NES) or Hsf family or group-specific (AHA motifs, OD). The DBD with its highly conserved 3D structure and central H2–T–H3 motif for HSE recognition as well as the HR-A/B region as OD represent the hallmarks of all eukaryotic Hsfs. Even the positioning of the intron in the DBD adjacent to the HTH motif is evolutionary conserved (Fig. 1, arrow heads).

Although the OD with its coiled-coil structure is present in all eukaryotic Hsfs, it comes in three different designs in plant Hsfs. This was utilized for distinguishing plant Hsfs into three major classes. The compact form of the OD characterizes the plant class B Hsfs and also all non-plant Hsfs, whereas representatives of the classes A and C share characteristic extensions of the linker regions between HR-A and B parts (see Section 2.2).

A type-specific sub-classification of plant Hsfs is facilitated by details concerning presence, position and sequence of NLS, NES, AHA motifs or activator regions. Remarkable in this context is that most of the functional domains described so far (see Sections 2.3 to 2.6) are characterized by fairly short motifs. Whether or not such a motif exerts its associated function may depend particularly also on its molecular context. Thus, there are clusters of basic amino acid residues, which are non-functional as NLS [39] or some of the canonical AHA motives, e.g. in HsfA5, are non-functional in plants ([57], see Section 4.6). Further evolutionary conserved sequence motifs, either adjacent to functional domains or isolated within the Hsfs provide additional evidence for classification. For practical purposes we summarize the above described information about shared features of Hsfs, i.e. the presence and characteristic of functional domains or motifs, their position within the protein as well as conserved sequence parts of unknown function, under the term signature sequences. To illustrate the point, two examples are given with tomato HsfA4b and HsfA5 (Fig. 2 and [57]). These signature sequences have proven useful to characterize the various Hsf types, e.g. HsfA1 type vs. A2 vs. A3 etc., or even subtypes such as Hsfs B2a vs. B2b or Hsfs A4a vs. A4b [30, 38, 57, 58]. Moreover, they are useful in assigning newly identified Hsfs to the appropriate type or sub-group. Giving the full set of signature sequences underlying our Hsf classification is certainly beyond the scope of this review. We will describe the compilation of the signature sequences together with our newly developed automated annotation pipeline for Hsfs elsewhere.

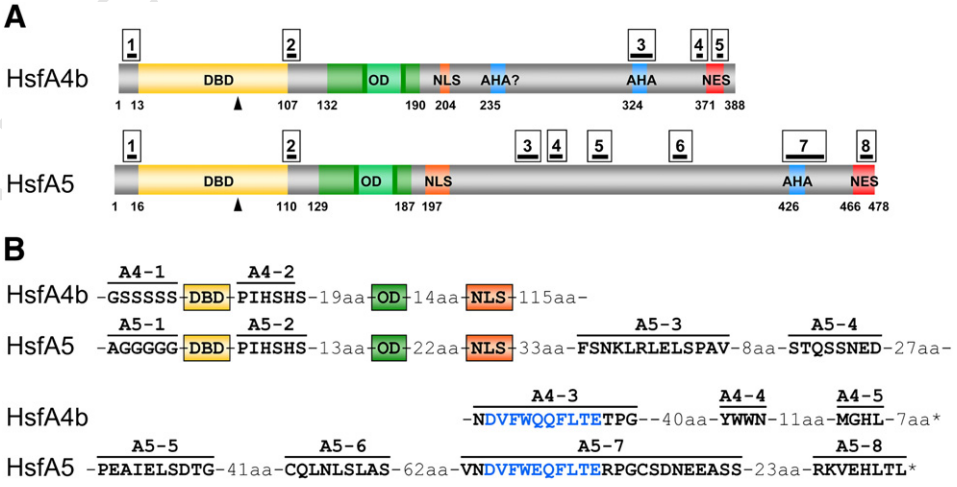


**Fig. 2.** Tomato Hsfs A4b and A5 with their signature sequences. A: Basic presentation, color code and abbreviations for functional motifs are similar to Fig. 1. B: The Hsf type-specific signature sequences and their positions relative to each other are indicated for tomato HsfA4b (A4-1 to A4-5) and HsfA5 (A5-1 to A5-8).

Please cite this article as: K.-D. Scharf, et al., The plant heat stress transcription factor (Hsf) family: Structure, function and evolution, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagrm.2011.10.002

## 3. Multiplicity of Hsfs

### 3.1. The plant Hsf family

The composition of the Hsf family in plants has so far been fully described only in few model species such as Arabidopsis and rice [1, 18, 59]. For example, *A. thaliana*, which served as the prototype for the Hsf family, has a set of 21 Hsf encoding genes with 15 members belonging to class A, 5 members to class B and one to class C (Table 1). However, recent analyses of Hsfs in other species indicated that both size and composition of the Hsf family is subject to evolutionary change. To get an overview of the Hsf composition across flowering plants we have extracted and characterized the Hsfs from 9 plant species with completely or almost completely sequenced genomes (Table 1). The references to the data sources are given in the legend to Table 1, and the complete nucleotide and amino acid sequences of the identified Hsfs are provided as information in our new data base (www.cibiv.at/services/hsf). Hsf families of 14 further plant species with far advanced sequencing of the genomes are compiled in Table S1, and the corresponding data are included in the new data base as well. Our survey revealed that the Hsf family of Arabidopsis is with only 21 members considerably small, and close to the smallest families observed so far in angiosperms with actually 18 or 19 Hsfs as found for *Ricinus*, *Vitis*, *Citrus* and *Carica* (Tables 1 and S1). The number of Hsfs in other plants species is typically higher with a current maximum of 52 Hsf genes identified in soybean.

The multiplicity of Hsfs in angiosperms is presumably the result of gene duplications and whole-genome duplications (WGD) at different points of evolution, followed by extensive gene loss (palaeodiploidization). Diversification of the remaining duplicates both in sequence and function led to the sets of Hsfs in contemporary angiosperms. Additional lineage-specific WGDs within the angiosperms presumably are the cause of varying numbers of Hsfs between different plant species. For example, in the evolution of the Arabidopsis lineage at least two additional rounds of WGD are assumed to have taken place approximately 60–70 and 23–43 Myr ago [60]. In the time since then most duplicates have been lost. In contrast, in the soybean lineage also two rounds of WGD have occurred, however, these events were more recent (~59 and ~13 Myr ago, respectively [61]). This may explain the much higher number of 52 Hsf encoding genes for soybean and the coexistence of 2–3 very closely related members of Hsfs in the individual groups (Table 1).

To get a better overview of the evolutionary relationships of the individual Hsfs detected and annotated by us, and to capture the evolutionary events that formed the contemporary Hsf families, we computed a phylogenetic tree for the 252 Hsfs. To warrant that only homologous sequences were used for the tree reconstruction, we limited the analysis to the N-terminal parts of the proteins containing the DBD and the OD (see double headed arrow on top of Fig. 1). To enhance readability of the resulting phylogenetic tree, we collapsed clades representing the same Hsf-type and sub-type, respectively (Fig. 3). The fully expanded tree is shown in the supplementary Fig. S1. The phylogenetic tree faithfully reflects the classification of the Hsfs based on the signature sequences (c.f. Section 2.7) indicating that the current annotation system of Hsfs by and large reflects the evolutionary relationships of the sequences. Although clearly separated in distinct groups, most of the Hsf-types are present both in eudicots and monocots. This has an interesting aspect for the evolution of the Hsf system in plants. Already the last common ancestor of the flowering plants had an Hsf family whose composition resembled that of the contemporary species. Preliminary data on the composition of the Hsf families in conifers (gymnosperms) indicate considerable deviations from the pattern found in angiosperms.

Despite the overall similarity between monocots and eudicots, there are also distinct differences. Representatives of HsfA9, HsfB3 and HsfB5 are confined to the eudicots and the corresponding types emerged presumably after the split of monocots and eudicots. The situation is yet unclear for HsfA9 function in monocots (see Section 4.8). The most marked difference between monocots and eudicots however is the substantially increased complexity of the HsfC group in monocots. Gene duplications on the monocot lineage led to the emergence of the monocot-specific types C1a, C1b, C2a and C2b. The functional consequences of this expansion remain yet to be determined.

In the phylogenetic tree (Fig. 3), we have followed the original nomenclature as worked out for the Arabidopsis Hsf family [1] and later applied also to the rice Hsf family [18, 59]. However, our increasing knowledge with now 9 plants and their full sets of Hsfs on the one hand, and more refined bioinformatic tools on the other hand led to few important changes and additions:

- Our earlier assignment of Hsfs in the closely related Hsf A2/A6/A7 group of rice ([18], see also [59]) had to be revised and adapted to the new complexity with three representatives for HsfA2 and two each for Hsfs A6 and A7.
- Because of lacking similarities with the seed-specific HsfA9 group of eudicots (see Section 4.8), the original rice HsfA9 was replaced into a new group HsfA8 together with the corresponding representatives of other monocots. It remains to be shown whether monocots also possess a seed-specific HsfA-type equivalent to HsfA9 (see Section 4.8) and whether the new monocot HsfA8 group is not only phylogenetically but also functionally related to the HsfA8 subtype of eudicots.
- Three unusual representatives of Hsf-like genes were identified in the tomato genome. They appear unique, and their expression and possible role within the Hsf family remains to be analyzed.

### 3.2. Non-plants Hsfs

The multiplicity of flowering plant Hsfs is in sharp contrast to the situation in most other organisms. The unique Hsfs in the yeast *Saccharomyces cerevisiae*, in nematodes and in *Drosophila* are not only required for the HS response. Thus, Hsf gene disruption in yeast is lethal even at normal growth temperatures [62, 63]. Although yeast contains three additional genes coding for Hsf-like proteins with conserved DNA-binding domain, i.e. Skn7, Mga1 and Sfl1 [37], none of these proteins is able to functionally replace the yeast Hsf. This provides the basis for testing heterologous Hsfs in yeast mutants with disruption of the *hsf1* gene [38, 64, 65]. In Drosophila, strains with a conditional lethal *hsf* allele survive, but they show abnormalities in oogenesis and early larval development [66]. Recent chromatin immunoprecipitation and microarray analyses confirmed that most of the *Drosophila* Hsf binding sites are actually not associated with HS genes, but with genes encoding developmental and reproductive proteins [67].

The major mammalian Hsfs responsive to stress induction are Hsf1 in cooperation with Hsf2 [19, 31]. However, both Hsfs have also essential functions in developmental processes, such as oogenesis, spermatogenesis or erythroid cell differentiation. In contrast to this, mammalian Hsf3 and Hsf4 have more specialized functions in stress response modulation and development [20, 31]. In addition, three Hsf-like proteins with unknown function were discovered in the human genome (HsfY1, HsfX1 and Hsf5). They contain the DBD but lack the characteristic HR-A/B region and other essential Hsf features [20].

## 4. Functional diversification and interactions of plant Hsfs

Our overall knowledge about the specific roles of different Hsfs in plants is still limited. But whenever analyzed in detail,



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**Table 1**
Plant Hsfs.

| Hsfs[a] | Arabidopsis At (21) [b] | Soybean Gm (52) | Poplar Pt (27) | Tomato Sl (24 + 3)[c] | Castor bean Rc (19) | Rice Os (25) | Brachypodium Bd (24) | Millet Sb (24) | Maize Zm (30) |
|---|---|---|---|---|---|---|---|---|---|
| HsfA1a | At4g17750 | Gm09g33920 | Pt0003s09370 | Sl08g005170 | Rc30054.t000017 | Os03g63750 | Bd01g01130 | Sb01g000730 | Zm2G115456 (1) |
| b | At5g16820 | Gm11g01190 | Pt0013s07730 | Sl03g097120 | Rc30073.t000085 | | | | Zm2G384339 (5) |
| c | At1g32330 | Gm01g01990 | Pt0001s02140 | Sl08g076590 | | | | | |
| d | At3g02990 | Gm16g13400 | | Sl06g072750 | | | | | |
| e | | Gm01g44330 | | | | | | | |
| HsfA2a | At2g26150 | Gm14g11030 | Pt0006s24330 | Sl08g062960 | Rc29739.t000001 | Os03g58160 | Bd01g05550 | Sb01g005250 | Zm2G132971 (1) |
| b | | Gm17g34540 | | | | Os07g08140 | Bd01g55630 | Sb02g004370 | Zm2G125969 (7) |
| c | | Gm04g05500 | | | | Os03g53340 | Bd01g08890 | Sb01g008380 | ZmBt085816 (1) |
| e | | | | | | | | | |
| HsfA3a | At5g03720 | Gm10g07620 | Pt0006s11680 | Sl09g009100 | Rc29092.t000013 | Os02g32590 | Bd03g44700 | Sb04g021490 | Zm2G059851 (5) |
| b | | Gm03g34900 | | | | | | | |
| c | | Gm13g21490 | | | | | | | |
| d | | Gm19g37580 | | | | | | | |
| HsfA4a | At4g18880 | Gm13g29760 | Pt0011s06820 | Sl03g006000 | Rc30026.t000048 | Os01g54550 | Bd02g49860 | Sb03g034630 | Zm2G118453 (8) |
| b | At5g45710 | Gm05g29470 | Pt0014s13780 | Sl07g055710 | Rc29636.t000015 | Os05g45410 | Bd02g18980 | Sb09g026440 | ZmAC206165 (6) |
| c | | Gm15g09280 | Pt0004s06090 | Sl02g072000 | | | | | ZmAC205471 (8) |
| d | | Gm08g12630 | | | | | | | |
| HsfA5a | At4g13980 | Gm05g28460 | Pt0017s08630 | Sl12g098520 | Rc29629.t000086 | Os02g29340 | Bd03g43710 | Sb04g020050 | Zm2G179802 (5) |
| b | | Gm08g11460 | Pt0001s32810 | | | | | | |
| HsfA6a | At5g43840 | Gm10g00560 | Pt0010s09210 | Sl09g082670 | Rc29844.t000068 | Os10g28340 | Bd03g26920 | Sb01g021490 | Zm2G010871 (1) |
| b | At3g22830 | Gm20g28870 | Pt0008s15740 | Sl06g053960/50 | | Os03g06630 | Bd01g74350 | Sb01g046350 | Zm2G165972 (1) |
| c | | Gm10g38930 | | | | | | | |
| HsfA7a | At3g51910 | Gm19g34210 | Pt0005s23640 | Sl09g065660 | Rc29883.t000010 | Os01g39020 | Bd02g41530 | Sb03g025770 | Zm2G005815 (3) |
| b | At3g63350 | Gm10g03530 | Pt0002s04900 | | | Os06g36930 | Bd01g37720 | Sb10g022340 | Zm2G173090 (9) |
| c | | Gm03g31380 | | | | | | | |
| HsfA8a | At1g67970 | Gm08g05220 | Pt0008s13620 | Sl09g059520 | Rc29968.t000004 | Os03g12370 | Bd01g69410 | Sb01g042370 | Zm2G118485 (1) |
| b | | Gm05g34450 | Pt0010s11490 | | | | | | Zm2G026742 (9) |
| HsfA9a | At5g54070 | Gm13g16510 | Pt0006s15050 | Sl07g040680 | Rc29912.t000123 | | | | |
| b | | Gm17g06160 | | | | | | | |
| HsfB1a | At4g36990 | Gm01g39260 | Pt0007s11030 | Sl02g090820 | Rc30115.t000024 | Os09g28354 | Bd04g32130 | Sb02g026590 | Zm2G002131 (2) |
| b | | Gm17g20070 | | | | | | | Zm2G139535 (7) |
| c | | Gm11g06010 | | | | | | | |
| d | | Gm05g20460 | | | | | | | |
| HsfB2a | At5g62020 | Gm09g26510 | Pt0012s13430 | Sl03g026020 | Rc30147.t000553 | Os04g48030 | Bd05g18680 | Sb06g025710 | Zm2G301485 (10) |
| b | At4g11660 | Gm11g02800 | Pt0001s08990 | Sl08g080540 | Rc30190.t000392 | Os08g43334 | Bd03g42130 | Sb07g025120 | Zm2G098696 (4) |
| c | | Gm10g38240 | Pt0015s13390 | | | Os09g35790 | Bd04g35780 | Sb02g030490 | Zm2G165272 (7) |
| d | | Gm16g32070 | | | | | | | Zm2G164909 (1) |
| e | | Gm20g29610 | | | | | | | |
| f | | Gm01g42640 | | | | | | | |
| HsfB3a | At2g41690 | Gm19g31940 | Pt0006s04770 | Sl04g016000 | Rc30006.t000013 | | | | |
| b | | Gm03g29190 | Pt0016s05680 | Sl10g079380 | | | | | |
| HsfB4a | At1g46264 | Gm04g04200 | Pt0002s12640 | Sl04g078770 | Rc30170.t000271 | Os08g36700 | Bd01g19900 | Sb02g026500 | Zm2G088242 (2) |
| b | | Gm20g08250 | Pt0009s07220 | Sl11g064990 | Rc28312.t000007 | Os07g44690 | Bd04g32050 | Sb02g040790 | ZmBT054148 (7) |
| c | | Gm06g04390 | Pt0014s02700 | | | Os09g28200 | Bd01g61620 | Sb01g034500 | ZmAC216247 (1) |
| d | | Gm14g04070 | Pt0001s28040 | | | Os03g25120 | | | |
| e | | Gm07g36370 | | | | | | | |
| f | | Gm14g09190 | | | | | | | |
| g | | Gm17g35980 | | | | | | | |
| h | | Gm02g44670 | | | | | | | |
| HsfB5a | | Gm13g24860 | Pt0004s04260 | Sl02g078340 | Rc29851.t000049 | | | | |
| b | | Gm01g34490 | Pt0011s05130 | | | | | | |
| HsfC1a | At3g24520 | Gm09g32300 | Pt0018s05770 | Sl12g007070 | Rc29912.t000252 | Os01g43590 | Bd02g44050 | Sb03g028470 | Zm2G086880 (8) |
| b | | Gm07g05910/20 | | | | Os01g53220 | Bd02g48990 | Sb03g033750 | ZmEU954042 (3) |
| c | | | | | | Os02g13800 | Bd03g08870 | Sb04g008300 | Zm2G089525 (3) |
| HsfC2a | | | | | | Os06g35960 | Bd01g38140 | Sb10g021800 | Zm2G105348 (5) |
| b | | | | | | | | | Zm2G118047 (9) |

[a] Web pages for blast searches and gene identification are: http://mips.helmholtz-muenchen.de/plant/genomes.jsp: Rice, *Brachypodium*, Millet (*Sorghum*), Arabidopsis, Tomato. http://comparative-legumes.org/: Soybean (*Glycine*). http://castorbean.jcvi.org/index.php: Castor bean (*Ricinus*). http://solgenomics.net/: Tomato. http://www.phytozome.net/search.php: *Brachypodium*, *Glycine*, Poplar, *Sorghum*. http://popcorn.maizegdb.org/main/index.php: Maize. http://rice.plantbiology.msu.edu/: Rice. http://signal.salk.edu/cgi-bin/tdnaexpress: Arabidopsis. http://www.arabidopsis.org: Arabidopsis. http://plants.ensembl.org/info/about/species.html: Arabidopsis, *Brachypodium*, Poplar, Rice, *Sorghum*, Maize.

[b] Organisms were abbreviated as follows: At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Gm, *Glycine max*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; Rc, *Ricinus communis*; Sl, *Solanum lycopersicum*; Sb, *Sorghum bicolor*; Zm, *Zea mais*. The total number of identified Hsf genes is given in brackets. Nomenclature: Usually the gene name includes also the chromosome number, e.g. At4g36990 for Arabidopsis HsfB1a indicates that the gene is on chromosome no. 4; for maize genes chromosome numbers are indicated in brackets; no chromosome numbers are available for *Ricinus*.

[c] Three Hsf-like (Hsfl) genes identified in tomato: Hsfl1 (Sl02g072060), Hsfl2 (Sl02g079180) and Hsfl3 (Sl11g008410).

there is a remarkable functional diversification, and the analyses of knock-out (KO) mutants indicate that usually the Hsfs cannot replace each other except within the subgroups, e.g. of HsfA1 (for details see Table 2 and Section 4.2). First, we will discuss functional diversification in more detail based on analyses of Hsf mutants (Section 4.1) and then we will focus more selectively on results obtained for individual Hsfs (Sections 4.2 to 4.8).

Please cite this article as: K.-D. Scharf, et al., The plant heat stress transcription factor (Hsf) family: Structure, function and evolution, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagrm.2011.10.002

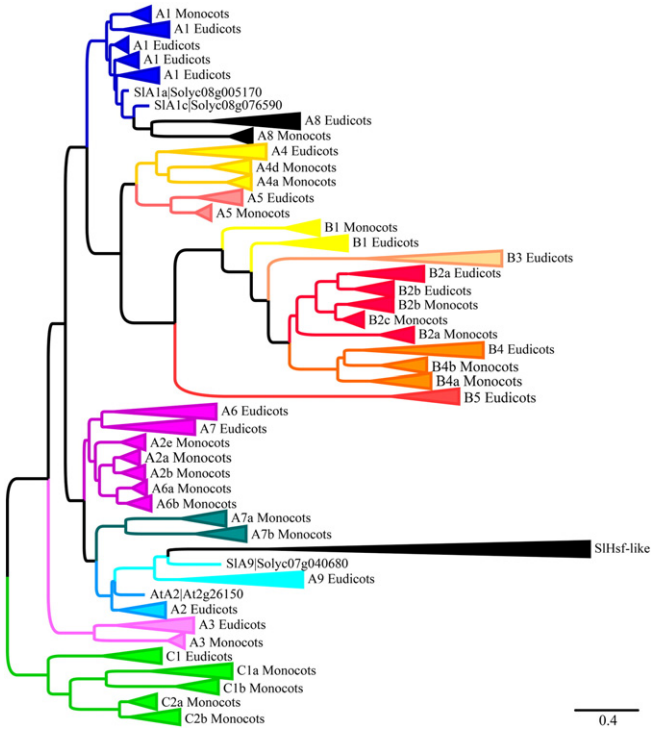


**Fig. 3.** Evolutionary relationship of the Hsfs from 9 plant species. The N-proximal parts of 250 Hsfs compiled in Table 1 containing both the DBD and the HR-A/B region (Fig. 1, double headed arrow), were aligned with MAFFT [184]. Without further processing the alignment was used for maximum likelihood tree reconstruction (RAxML v.7.2; [185]) using the PROTGAMMAILGF model of sequence evolution. For reasons of clarity, the tree was arbitrarily rooted with the class C Hsfs. Moreover, we collapsed clades of sequences annotated with the same Hsf type, where applicable. The fully expanded tree is provided for more detailed inspection in supplementary Fig. S1. Scale bar for the branch length represents 0.4 expected substitutions per site.

### 4.1. Hsf mutants and phenotypes

Details of Hsf function were usually elaborated by testing corresponding mutant forms in transient expression assays after PEG mediated transformation of protoplasts or particle bombardment of epidermis cells of sunflower embryos [68] or Arabidopsis leaves [55]. The experiments led to the identification of various functional motifs/domains, such as the NLS [39], NES [38, 41], AHA motifs [38, 42–44], the role of the OD for Hsf interactions [35, 40, 57, 69, 70] and of chaperones for the control of Hsf function [71–73].

For analyses of Hsf mutant phenotypes *in planta* there is a unique collection of mostly T-DNA insertion lines of Arabidopsis publically available from the SALK Institute San Diego (http://signal.salk.edu/cgi-bin/tdnaexpress). These KO mutant lines lacking individual Hsfs or combinations of them obtained by crossing form the basis of most investigations compiled in Table 2. In addition, a number of Hsf overexpression lines (OE) of Arabidopsis, especially of HsfA2 (Table 2, group I, nos. 7–9) and HsfA9 (no. 15), helped to clarify the particular functions of these Hsfs for thermotolerance and seed maturation, respectively. Further interesting insights were obtained by generating transgenic plants expressing dominant negative forms of Hsfs obtained either by deletion of the CTD or by fusion with a chimerical repressor motif (group I, nos. 3, 10 and 12). In few cases loss-of-function (LOF) mutants were identified as a result of mutant screens (group I, no 23). It is remarkable that some Hsf KO or LOF mutant lines show clear phenotypes, indicating that the lack of function of these Hsfs cannot be compensated by others (nos. 6, 13, 14, and 20). In other cases, however, only double KO mutants (no.18) or even quadruple KO mutants gave clear negative effects (no. 5).

We complemented the data shown in Table 2 by a group of selected mutants which provide interesting insights into Hsf function, because either Hsf interacting proteins or components of stress signal transduction are affected (Table 2, group II, nos. 1 to 13).

### 4.2. Identification of HsfA1a as master regulator in tomato

An essential clue to the functional diversification within the tomato HsfA1 group came from analyses of transgenic plants with knock-down of HsfA1a expression as a result of posttranscriptional gene silencing (cosuppression, CS plants). These plants were similar to wild type plants in all major developmental parameters but were extremely sensitive to elevated temperatures, because HS-induced synthesis of Hsfs A2 and B1 as well as that of chaperones was practically eliminated by the knock-down of HsfA1a expression [74]. Despite the complexity of the Hsf family (Table 1), HsfA1a appears to have a unique function as master regulator for acquired thermotolerance, and cannot be replaced by any other Hsf. It is responsible for triggering the HS response and later on, by interaction with Hsfs A2 and B1 in a functional triad, affects different aspects of the HS response and recovery (Sections 4.3 and 4.4).

The composition of the Hsf families of tomato and Arabidopsis is largely congruent (Table 1). However, no comparable role as master regulator could be identified for any of the four AtHsfA1 [75, 76]. KO mutants with single knock outs of Hsfs A1a, A1b, A1d or A1e, as well as double or triple KO mutants had no marked defects in the overall HS response and long-term thermotolerance level of Arabidopsis [75, 76]. However, transcriptome analysis of double KO mutants indicated that these Hsfs have a certain role for the HS-induced transcription of a subset of genes, which includes not only genes encoding small heat shock proteins (sHsps), Hsp70 and Hsp101, but also genes encoding some Hsfs like HsfA2, HsfA7a, HsfB1 and HsfB2a, as well as genes encoding HS-induced metabolic enzymes, such as inositol-3-phosphate synthase2 (Ips2) and galactinol synthase 1 (GolS1). The search for the "master regulator" of the Arabidopsis HS response was successful when a quadruple KO mutant with complete lack of all four HsfA1 representatives was tested [77]. However, in this case the mutant plants were not only seriously impaired in the HS response and acquired thermotolerance but had also marked developmental defects.

The apparent differences between tomato with a single master regulator (HsfA1a, [74]) and Arabidopsis with the HsfA1 group [77] are striking. However, it cannot be excluded that, in fact, tomato is closer to the Arabidopsis situation than thought before. The cosuppression situation in tomato, with siRNAs generated due to inverted repeat insertion in the genome, might have affected not only the expression of HsfA1a as tested in the publication of Mishra et al. [74]. The expression of the other members of the tomato HsfA1 group could not be tested at the time of the experiments. Although the normal phenotype and development of the tomato CS-plants argues against such an interpretation, the case needs reinvestigation.

### 4.3. HsfA2 as HS-induced enhancer of thermotolerance

HsfA2 is structurally and functionally similar to HsfA1 [43], but it is only expressed in stressed plants. However, it belongs to the most strongly induced proteins in tomato, Arabidopsis and rice accumulating to high levels in plants exposed to long-term HS or repeated cycles of HS and recovery [40, 42, 74, 78–81]. The crucial effects of HsfA2 for high levels of induced thermotolerance evidently depend not only on the abundance of this Hsf in stressed plants but also on heterooligomerization with HsfA1. Together, the two proteins form a type of superactivator complex for Hsp encoding genes, whose activity is much higher than that of the two Hsfs individually (Fig. 5 and refs. [40, 69]). The superactivator function of the tomato HsfA1/A2 heterooligomers very likely reflects the combination of the two type of activation domains with their different types and patterns of AHA motifs. It is tempting to speculate that the observed interaction between the Hsfs of the



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Arabidopsis HsfA1 group [82] could have similar combinatory effects, because the C-terminal activation domains of the four representatives are quite different [38].

In addition to the effects of HsfA2 on the thermotolerance level, the comprehensive analyses of Arabidopsis HsfA2 KO lines indicated a broader role for expression of general stress-related, non-chaperone encoding genes like *GOLS1* (*galactinol synthase 1*) or *APX2* (*ascorbate peroxidase 2*) [79, 81, 83, 84]. In support of this, KO plants were sensitive to HS, high light, oxidative stress and anoxia, whereas Arabidopsis plants with overexpression of HsfA2 showed not only higher levels of thermotolerance but also increased resistance to salt/osmotic stress [85, 86], oxidative stress [84] and anoxia [87]. In summary, HsfA2 can be considered as one of the key regulators of plant stress response protecting also against oxidative damage of organelles and subsequent cell death [84]. Finally, it is worth noticing that expression of HsfA2 together with chaperones Hsp90, Hsp70 and Hsp17-CII was found as integral part of anther development in tomato, indicating that preformed chaperones may be important to protect maturing and germinating pollen from heat damage [7, 88, 89].

### 4.4. Tomato HsfB1 acts as synergistic coactivator of HsfA1a

In contrast to class A Hsfs, a considerable number of Hsfs assigned to classes B and C have no evident function as transcription activators on their own [35, 38, 90]. On the contrary, a highly conserved -LFGV- tetrapeptide in all class B Hsfs forms the core of a repressor domain (see Section 4.5). However, under certain conditions of appropriate promoter architecture, the HS-induced tomato HsfB1 can act as coactivator cooperating with class A Hsfs, such as HsfA1a. The two Hsfs assemble into an enhanceosome-like complex, necessary to recruit the plant CREB binding protein (CBP) ortholog histone acetyl transferase HAC1. Formation of this ternary complex results in strong synergistic activation of reporter gene expression [35]. Moreover, HsfB1 also cooperates with other transcriptional activators controlling house keeping gene expression. HsfB1 might help to maintain and/or restore expression of housekeeping genes during HS. The intriguing interactions between tomato Hsfs A1a, A2 and B1 as a functional triad and the role of chaperones for regulation of the different stages of the HS response are summarized in Section 5.2.

### 4.5. Repressor function of class B Hsfs

The lack of activator functions in class B Hsfs led to the identification of a repressor domain in the C-terminus [54]. Amino acid sequence comparison between many members of the B class Hsfs identified an almost invariant -LFGV- tetrapeptide motif adjacent to basic clusters, which apparently form the core of the repressor domain. Similar -LFGV- motifs are found also in other plant transcription factors known to have repressor functions, e.g. ABI3/VP1, AP2/ERF, MYB and GRAS [55]. However, the role of the conserved tetrapeptide motif is far from clear, because appropriate mutant analyses have not been undertaken, and the putative corepressor remains to be identified. For their experimental tests Ikeda and Ohme-Takagi used ABI3/VP1 and only demonstrated that two flanking hydrophobic residues (underlined) are crucial for function (-LRLFGVNM-); but changes in the core motif were not tested.

Interestingly, analyses with Arabidopsis *hsfB1/hsfB2b* double KO plants indicated a role of class B Hsfs for repression of HS gene expression during recovery and of pathogen resistance by control of defensin Pdf1.2 gene expression. Results indicate that due to the loss of the repressor function in the double KO mutant plants, Pdf1/2 mRNA levels were highly up-regulated. The effect seems to be gene specific, because HsfA2 mRNA levels were barely affected [56]. But it is interesting to notice that Pdf1.2 encoding genes are among the HS-inducible genes in Arabidopsis [91].

### 4.6. HsfA5 acts as specific repressor of the antiapoptotic HsfA4

An intriguing functional peculiarity was reported for two phylogenetically related class A Hsfs of tomato and Arabidopsis (Fig. 2). Despite structural similarities, HsfA4 act as potent activators of HS gene expression, whereas group A5 Hsfs are inactive and inhibit HsfA4 activity. Evidently, HsfA5 interferes specifically with the active oligomeric state of HsfA4 and, hence, with its DNA-binding capacity [57]. Interestingly, neither HsfA5 nor A4 interact with HsfA1 or HsfA2 and vice versa, HsfA1 cannot interact with Hsfs A4 or A5. However, the molecular details of this specificity of the OD have yet to be clarified. The OD of HsfA5 alone is necessary and sufficient to exert the repressor effect on HsfA4. Pull-down assays and yeast two-hybrid interaction tests have shown that HsfA4/HsfA5 heterooligomer formation is preferred to homooligomer formation of both Hsfs [57].

Despite the presence of a conserved *bona fide* AHA motif, e.g. -DFWEQFLTE- for AtHsfA5, there is no measurable activator function of HsfA5 in plants. This intriguing observation once more underlines the importance of the molecular context of a given motif. Interestingly, tests in yeast monohybrid reporter assays indicate a normal transcriptional activator function of the CTD of AtHsfA5, if fused to the yeast Gal4-DBD, i.e. in the heterologous context. Moreover, as expected, this AHA motif can be inactivated by W > A mutation [38].

The role of HsfA5 as repressor of HsfA4 is intriguing because there are experimental findings about tissue and stress specific high expression levels (see Section 4.9) and specialized functions of Hsfs A4:

(i) A rice HsfA4d mutant (*spl7*) with an W > C transition in the ß1 strand of the DBD showed spontaneous necrotic lesions in mature leaves due to a hypersensitivity to mild stress conditions [92]. Unfortunately, the role of this amino acid exchange on DNA binding or other HsfA4d functions was not further studied.

(ii) Transgenic Arabidopsis plants harboring a dominant negative mutant form of HsfA4a are negatively affected in their response to oxidative stress due to decreased levels of ascorbate peroxidase 1 (Apx1) levels [93].

(iii) Wheat and rice HsfA4a, but not HsfA4d, conferred cadmium (Cd) tolerance to Cd-sensitive yeast strains and to rice plants with OE of wheat HsfA4a. In agreement with these observations, HsfA4a transcript levels were highly increased in roots of wheat and rice exposed to Cd stress. Moreover, rice KO lines lacking HsfA4a were found to be Cd-hypersensitive [94]. Results indicate interesting peculiarities in the ß1, ß2 strands of the HsfA4a DBD as basis for selective promoter recognition. Compared to HsfA4d only two amino acid residues are changed.

### 4.7. HsfA3 as part of drought stress signaling

The functional anatomy of tomato HsfA3 is basically similar to HsfA1a and HsfA2, except that the C-terminal activator region appears more diffuse with a pattern of conserved tryptophane residues (Section 2.5, [44, 73]). A recent investigation showed that the drought and HS-induced expression of HsfA3 in Arabidopsis depends on the DREB2A transcription factor (dehydration-responsive element binding protein 2A), and this also holds true for genes encoding Hsp18.1-CI, Hsp26.5-MII and Hsp70 [95, 96]. Overexpression of DREB2A or DREB2C led to the induction of HsfA3 and consequently of other HS-related genes. This was accompanied by higher tolerance to HS treatments, whereas DREB2A KO mutants showed reduced thermotolerance [96–98]. Similar results were obtained by overexpression of the Zea mays DREB2A in Arabidopsis [99].

### 4.8. HsfA9 controls Hsp expression during seed development

The unique role of HsfA9 during seed development represents another case of functional diversification. HsfA9 was characterized



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**Table 2**
Overview of mutant lines with impaired Hsf expression/function.

| | | Genes, constructs | Mutation[a], mutant lines | Phenotypes | Ref. |
|---|---|---|---|---|---|
| Group I | | | | | |
| *Arabidopsis. thaliana* | | | | | |
| | 1 | *Hsfs A1a, A1b*<br>*HsfA1a/b* | KO, TDI;<br>DKO, TDI | No influence on Hsp expression levels in single KO mutants;<br>In DKO line delayed expression of Hsp18.1-CI as well as Hsfs A7a, B1, and B2a genes during early HSR; no influence on BT and only mild effects on AT; no influence on plant morphology. | [75, 186] |
| | 2 | *Hsfs A1d, A1e*<br>*HsfA1d/e* | KO, TDI<br>DKO, TDI | No marked influences on Hsp expression levels in single KO mutants;<br>Reduced induction of Hsfs A2, A7a, A7b, B1 and B2a transcript levels under HS and HL; no effects on BT but AT reduced; no differences in phenotype. | [76] |
| | 3 | *HsfA1d-SRDX*<br>*HsfA1e-SRDX* | DN<br>DN | Fusion of the ear-like repressor motif SRDX to C-terminus; induction of HsfA2 transcript levels reduced under HS and HL in both mutant lines; no changes of phenotype. | [76] |
| | 4 | *HsfA1aTK*<br>*HsfA1bTK*<br>*HsfA1dTK*<br>*HsfA1eTK* | TKO of A1b,d,e<br>TKO of A1a,d,e<br>TKO of A1a,b,e<br>TKO of A1a,b,d | Complete loss of TT in HsfA1eTK mutant seedlings and adult plants only; TT partially impaired in bTK, but not affected in aTK or dTK mutants; Hsp up-regulated in aTK and dTK, but reduced in bTK. | [77] |
| | 5 | *HsfA1QK* | QKO of all four A1 Hsfs | Complete loss of TT in mutant seedlings and adult plants; impaired tolerance against salt, osmotic and oxidative stresses; seedlings display diverse phenotypes and growth retardation in correlation with reduced Hsp90 levels. | [77] |
| | 6 | *HsfA2* | KO | Reduced transcript levels for Hsp70-5, Hsp18.1-CI, Hsp22-ER, Hsp25.3-P, Hsp26.5-MII, Hsa32 and Apx2; reduced long-term AT but no effect on short-term TT; no increased sensitivity to HL stress; no morphological or developmental phenotypes; protoplasts of mutant lines accumulate higher levels of ROS during HS and show severe mitochondrial dysfunction and reduced cell viability; mutant TT phenotypes rescued by HsfA2 complementation. | [79, 80, 83, 84, 187] |
| | 7 | *HsfA2* | OE | Constitutive up-regulation of putative HsfA2 target genes but only mild effects under combined stress conditions (HS, HL, and oxidative stress). | [83] |
| | 8 | *HsfA2* | OE | Enhanced anoxia tolerance with enhanced expression of anoxia response genes (*SUS4*, *ADH*) and HsfA2 target genes; cross-acclimation to anoxia through mild HS pre-treatment is impaired in HsfA2 KO mutant plants. | [87] |
| | 9 | *HsfA2*<br>*HsfA2* | OE, *P35S*<br>OE, *PEl2Ω* | HsfA2 OE lines using *P35S* promoter for ectopic expression control show enhanced tolerance to salt and osmotic stress; dwarfism by HsfA2 OE under control of *PEl2Ω*, but not with *P35S*; enhanced callus formation and acceleration of callus growth | [85] |
| | 10 | *HsfA2ΔC264* | DN | Reduced BT and AT, as well as reduced expression levels of putative HsfA2 target genes after HS induction. | [85] |
| | 11 | *HsfA3* | KO; TDI<br>KD, RNAi | Reduced levels of Hsps (Hsp101, sHsps); reduced BT and AT, no morphological phenotype. | [96] |
| | 12 | *HsfA4a* | DN | Ectopic expression of HsfA4a with deleted C-terminal AD; prevents expression of Apx1 and Zat12 under light stress; increased transcript levels of endogenous HsfA4a. | [93] |
| | 13 | *HsfA4c* | KO, TDI (*rha1*) | Loss of right-handed root slanting and reduced gravitropism; shorter root and shoot size; shorter siliques; reduced production of lateral roots; reduced sensitivity to 2,4-D, auxin transport inhibitors, and ethylene. | [188] |
| | 14 | *HsfA7a*<br>*HsfA7b* | KO<br>KO | Strongly reduced AT for HsfA7a KO lines, although highly similar with HsfA2, both HsfA7a and A7b KO lines have no TT phenotype comparable to HsfA2 KO lines, no morphological phenotype. | [80, 189] |
| | 15 | *HsfA9* | OE | Ectopic expression confers constitutive Hsp expression in leaves; loss of HsfA9 and seed specific Hsp expression in *abi3-6* mutants; in contrast, ectopic expression of ABI3 results in HsfA9 and Em1 expression in seedlings in the presence of ABA. | [101] |
| | 16 | *HsfB1* | KO, TDI | No HS phenotype | [56, 189] |
| | 17 | *HsfB2b* | KO, TDI | No HS phenotype | [56, 80] |
| | 18 | *HsfB1/B2b* | DKO, TDI | No obvious phenotype in TT, growth or fertility but up-regulation of defensin encoding genes Pdf1.2a and 2b results in increased resistance to pathogen infection. | [56] |
| *Solanum lycopersicum* | | | | | |
| | 19 | *HsfA1a* | OE | Enhanced expression of HS-inducible Hsfs and Hsps as well as of BT and AT; no obvious morphological and developmental phenotypes at control temperature. | [74] |
| | 20 | *HsfA1a* | CS | Strongly reduced expression of HS-inducible Hsfs and Hsps combined with loss of BT and AT in adult plants and during fruit ripening; no obvious morphological and developmental phenotypes under control conditions; HsfA1a as master regulator. | [74] |
| *Oryza sativa* | | | | | |
| | 21 | *HsfA2a* | OE in *A. thaliana* | Constitutive expression of a sub-fraction of AtHsfA2 target genes; enhanced BT and AT in rosette leaves, inflorescence stems and enhanced TT of germinating seeds; increased salt tolerance; slightly retarded growth and dark green leaves. | [86] |
| | 22 | *HsfA4a*<br>*HsfA4a* | KD, TDI<br>KO, TDI | Reduced Cd tolerance in KD mutants with TDI in promoter region; no growth phenotype<br>No obvious phenotype by for KO mutants with TDI in exon 2. | [94] |
| | 23 | *HsfA4d* | LOF, (*spl7*) | Mutation of the conserved W>C in ß1 of the DBD; enhanced leaf spot (lesion-mimic) phenotype and increased susceptibility to several pathogens; HsfA4d as anti-apoptotic factor in pathogen defence response. | [92] |
| *Triticum aestivum* | | | | | |
| | 24 | *HsfA4a* | OE in *O. sativa* | Enhanced expression of metallothionein gene MT-I-1a, increased Cd tolerance; TT not altered; slightly retarded growth in non-stressed seedlings; similar effects also in Hsf-deficient yeast cells when transformed with HsfA4a but not with HsfA4d. | [94] |
| *Helianthus annuus* | | | | | |
| | 25 | *HsfA9* | OE in *N. tabacum* | HsfA9 with seed specific *PDS10* leads to enhanced expression of seed specific sHsps but not Lea proteins; increased seed BT and longevity.<br>HsfA9 with *P35S* causes ectopic expression of seed-specific sHsps in vegetative tissues and leads to increased dehydration-tolerance; no developmental or growth phenotypes. | [102, 190] |
| | 26 | *HsfA9-SRDX* | DN in *N. tabacum* | Seed-specific expression of the HsfA9 repressor form causes reduced sHsp accumulation but no effect on Lea protein levels; reduced dehydration tolerance and longevity; survival of embryos to developmental desiccation was not impaired. | [104] |

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t2.35 **Table 2** (*continued*)

| | | Genes, constructs | Mutation[a], mutant lines | Phenotypes | Ref. |
|---|---|---|---|---|---|
| t2.37 | Group II | | | | |
| | Other mutants influencing Hsf expression/function | | | | |
| t2.38 | 1 | *AtROF1* *AtROF2* | KO, TDI OE KD, RNAi | Reduced Hsp levels during recovery from HS; impaired long-term AT; results point to regulation of HsfA2 activity via Hsp90 interaction; no constitutive expression of Hsps in ROF1 OE plants, but mutant plants show enhanced long-term AT, no obvious phenotypes observed in any of the mutant plants under normal growth conditions. | [121, 122] |
| t2.39 | 2 | *AtHSBP* | KO, TDI OE | Enhanced Hsp accumulation after HS; no influence on BT but increased AT; earlier flowering, shorter siliques, and increased seed abortion. In HSBP OE plants reduced Hsp accumulation and reduced TT but no obvious phenotype. | [126] |
| t2.40 | 3 | *ZmHSBP1* | null allele (*emp2*) by Tn insertion | Truncated EMP2 transcripts accumulate in mutant plants; increase of Hsp expression; loss of HSR attenuation leads to embryo abortion. | [125, 191] |
| t2.41 | 4 | *AtCBK3* | KO, TDI OE | Down-regulation of Hsf DNA-binding activity and Hsp expression in KO lines; reduced BT. Up-regulated Hsp expression and BT in OE mutant plants; no obvious phenotypes; CBK3-HsfA1a interaction *in planta*. | [175] |
| t2.42 | 5 | *AtCaM3* | KO, TDI OE | Heat sensitive phenotype in KO mutant plants rescued by ectopic CaM3 expression. Enhanced BT in CaM3-GUS transgenic plants with increased Hsf DNA-binding activity and sHsp expression. | [177] |
| t2.43 | 6 | *AtNOA1* | KO, TDI (*noa1/rif1*) | Lower NO levels correlate with increased heat sensitivity; phenotype rescued by ectopic expression of CaM3; results indicate NO as component involved in HS signaling together with CaM3 and CBK3. | [178] |
| t2.44 | 7 | *AtAPX1* | KO, TDI | Increased expression of Hsfs A4a, A4b, and B1 induced by light stress treatment; enhanced $H_2O_2$ levels and Hsp expression; enhanced protein oxidation. | [93, 192] |
| t2.45 | 8 | *AtDREB2A CA* *AtDREB2A* | GOF KO, TDI | Deletion of negative regulatory domain in DREB2A CA(Δ136-165) leads to a constitutively active form; expression of salt, draught, and HS-related genes, including HsfA3. DREB2A up-regulated genes are down-regulated in *DREB2A* KO mutants under stress conditions, reduced TT. | [95–97, 193] |
| t2.46 | 9 | *AtDREB2C* | OE | Specific transactivation of *DRE* dependent HsfA3 transcription by DREB2C, strong expression of HsfA3 results in Hsp synthesis and increased AT. | [98] |
| t2.47 | 10 | *HaDREB2* | OE in *N. tabacum* | Expression with seed specific promoter; no influence on Hsp expression and BT during seed germination, no effects on seed longevity; enhanced BT and seed longevity in combination with HaHsfA9 overexpression. | [103] |
| t2.48 | 11 | *ZmDREB2A* | OE in *A. thaliana* | Enhanced expression of HsfA3 in OE plants, enhanced tolerance to draught and HS, OE under control of *P35S* leads to reduced growth of rosette leaves and delay in bolting time, which was diminished in OE plants under control of the cold and draught inducible *PRD29A*. | [99] |
| t2.49 | 12 | *AtMBF1c* | KO, TDI OE | KO seedlings deficient in BT but not influenced in AT, HS induction of HsfA2, Hsps and Apx1 was not affected. Increased BT in OE plants; elevated expression of oxidative stress response factor Zat12 and HsfB2a, HsfB2b, and DREB2A was impaired. | [181, 194, 195] |
| t2.50 | 13 | *AtWRKY39* | KD, TDI OE | Down-regulation of salicylic acid (SA) related *PR1* and *MBF1c* genes in KD mutants, increased susceptibility to HS with reduced germination, decreased survival and elevated electrolyte leakage WRKY39 OE led to increased BT, no influence on Hsp101, Hsp70 or Apx1 expression. | [160] |

[a] Abbreviations: KO, knock-out; KD, knock-down; DKO, double KO; TKO, triple KO; QKO, quatro KO; CS, cosuppression; OE, overexpression; DN, dominant negative; GOF, gain of function; LOF, lost of function; TDI, T-DNA insertion, RNAi, RNA interference; Tn, transposon; *P*, promoter: *35S*, constitutive Cauwliflower mosaic virus (CaMV) *35S* gene promoter; *DS10*, seed specific *Δ9-stearoyl-(acyl carrier protein) desaturase* promoter; *El2Ω*, chimeric *P35S* with tobacco mosaic virus (TMV) omega sequence; *RDA29*, stress inducible *RDA29* promoter; DRE, drought responsive element; HS, heat stress; HL, high light; HSR, HS response; TT, thermotolerance; BT, basal TT; AT, acquired TT.

as a specialized Hsf for embryogenesis and seed maturation in sunflower and Arabidopsis [68, 100, 101]. In developing Arabidopsis seeds the expression of HsfA9 is controlled by transcription factor ABI3 (abscisic acid-insensitive 3) [101]. Ectopic expression of HsfA9 caused formation of sHsps and Hsp101 in leaves under unstressed conditions [101], and overexpression of sunflower (*Helianthus annuus*, Ha) HsfA9 alone or together with HaDREB2 in tobacco seeds enhanced the accumulation of Hsps and improved seed longevity [102, 103]. Thus, the HS-independent role of HsfA9 probably results from its cooperation with other developmental transcription factors like ABI3 or DREB2 formed during seed maturation [100, 101]. On the other hand, sunflower HsfA9 was shown to physically interact with the IAA27 repressor of auxin response, i.e. the intriguing role of HsfA9 in seed maturation appears to be embedded into the hormonal control networks dominated by abscisic acid (ABA) and auxins [70]. Interestingly, expression of a dominant negative form of HaHsfA9 in tobacco plants resulted in drastically reduced levels of seed-specific sHsps with only minor effects on seed maturation and germination [104]. The latter results indicate that, in contrast to earlier assumptions, HsfA9 function is not essential for development of seed desiccation stress tolerance. In view of the evident lack of HsfA9 in monocots, it is intriguing that rice microarray data indicate very high levels of OsHsfA1a mRNA in seeds but not in other tissues (http://bar.utoronto.ca). The functional significance of this has to be shown. Respectively, it is interesting to notice that in contrast to all other eudicots investigated so far with usually a single HsfA9 encoding gene, *Eucalyptos grandis* (*Myrtaceae*) contains at least 17 closely related HsfA9 encoding genes in addition to the normal set of 20 other Hsfs (Table S1). It will be interesting to investigate the expression patterns of these HsfA9 genes and to elucidate the functional significance of this surprising expansion of the A9 group.

### 4.9. Diversification by expression

Although detailed functional analyses of Hsfs are limited to the examples described above, comprehensive microarray expression data compiled in the AtGenExpress data base (https://www.genevestigator.com; http://jsp.weigelworld.org/expviz/; http://bar.utoronto.ca) provided the basis for a more detailed view on the Hsf transcriptome of Arabidopsis during development as well as during abiotic and biotic stress responses [105–108]. The most striking results can be summarized as follows:

(i) Hsf expression patterns in different organs indicate that the four members of the HsfA1 group are constitutively expressed at low levels in most organs. As already mentioned, HsfA9 is exclusively expressed during seed maturation, and transcripts of Hsfs A1a, A4c and A5 are mainly found in developing anthers and/or pollen. Transcripts of Hsfs A4c, A7a, B1 and C1 are enriched in roots, and those of Hsfs A4c, A8 and B2a are higher in leaves.
(ii) Irrespective of the tissue, expression of HsfA2 and, to a certain extent also of Hsfs A1d, A4a, A4c, A7a, A7b, A8, B1, B2a, B2b, B4, and C1 are induced by different abiotic stressors, particularly in roots.
(iii) Transcripts coding for Hsfs A1e, A3, A4a, A4c, A6b, A8, B2a, and C1 are particularly prominent in osmotic, salt and cold stress samples.



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(iv) Transcripts coding for Hsfs A2, A4a, A8, and B1 are induced in response to various biotic stressors.

Obviously, mRNA levels cannot be used to draw immediate conclusions about protein levels. However, they can point out directions of further investigations. The corresponding protein data for the Arabidopsis Hsfs are only available for HsfA2 and HsfA9 [79, 101]. The summary of changing mRNA levels from the AtGenExpress sources makes it very likely that at least part of the Hsf diversity results from their particular expression patterns during different stress and developmental situations. Unfortunately, data sets of comparable complexity are not available for any other plant (http://bar.utoronto.ca). However, complex changes of Hsf mRNA levels during development as well as heat, cold and oxidative stress were also reported for rice [59, 109, 110].

## 5. Control of Hsf activity

### 5.1. Mammalian Hsf1

Because of the central role of chaperones in many aspects of molecular cell biology and human diseases, structure and function of Hsfs, especially human Hsf1, were extensively studied. The mammalian system serves as an excellent example for the multilevel control of the stress response but also of developmental processes under the control of Hsf1 [15, 19, 31]. Thus, we want to emphasize similarities and differences between mammals and plants by briefly summarizing results from mammalian cells (Fig. 4).

We can discriminate four distinct states of human Hsf1:

(i) Similar to steroid receptors of mammals [17], the inactive and hypophosphorylated Hsf1 exists in cytoplasmic complexes with the Hsp90 complex.

(ii) Upon stress treatment, e.g. as a result of imbalanced protein homeostasis (cytosolic protein response), the release of Hsf1 from the chaperone complex allows trimerization, nuclear import and binding to HSE containing DNA sequences. This process is connected with increased phosphorylation and sumoylation in the repressor region C-terminal of the HR-A/B domain. It is a matter of speculation that, similar to the situation in Drosophila, Hsf1 binds preferably to HSE in open chromatin regions characterized by appropriately modified nucleosomes and the RNA polymerase II (RNAPII) machinery [111], i.e. HS-inducible genes exist in a pre-activated state. Interestingly, Hsf1 was reported to mediate genome-wide decrease of histone acetylation which may indicate the profound transcriptional reprogramming upon HS [112].

(iii) Transcription activation involves removal of the sumo residue as well as further phosphorylation of Hsf1 trimers and interaction with components of the RNAPII machinery (SWI/SNF, Mediator complex) to allow the transition of the RNAPII complex to the elongation mode and entry of a new RNAPII in the initiation form.

(iv) Attenuation (inactivation) of Hsf1 results from binding of the Hsp70 machinery and dephosphorylation. In this last step, Hsf1 is acetylated in the DBD [113]. The chaperone binding is considered as a type of feed back control of Hsf activity after the cytosolic levels of free chaperones are restored. In its attenuation function, Hsp70 interacts with CoREST, a general corepressor and component of histone deacetylase complexes [114].

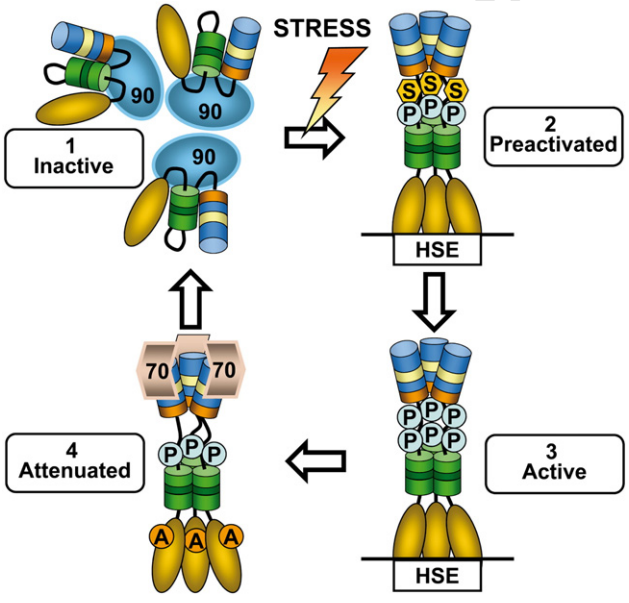


**Fig. 4.** Model of the human Hsf1 activity cycle (according to Akerfelt et al. [31]; modified). For explanation of the four states in the Hsf1 cycle see text. The color code of DBD, OD and C-terminal activator domain is the same as used in Fig. 1. 70 and 90 represent the Hsp70 and Hsp90 chaperone machines; P, S, A, phosphorylation, sumoylation and acetylation sites of Hsf1 occupied during different states of the cycle (details see text).

### 5.2. Control of Hsf activity in plants

Early observations with band shift assays and tomato nuclear extracts confirmed HS-inducible binding of Hsfs, very likely HsfA1a, to HSE containing oligonucleotides [78]. Similar to the situation in mammalian cells (see Section 5.1), the molecular basis of Hsf activation is assumed to involve release from Hsp90/Hsp70 chaperone complexes as a result of the cytosolic protein response ([72, 115, 116]. The role of both chaperone systems for activity control and stability of HsfA1a, HsfB1 and HsfA2 are complex, and the underlying interactions and targeted functions appear to be highly specific for both partners, Hsfs and chaperones respectively ([72, 116–120]. Further details are summarized in Fig. 5.

In addition, activity and availability of the dominant HsfA2 in long-term stressed cells is under control of small Hsps. Hsp17-CII directly interacts with HsfA2 forming inactive complexes which finally accumulate in giant protein aggregates (heat stress granules, Fig. 5C and refs. [40, 71]). Release of HsfA2 from the inactive storage sites requires Hsp17-CI and probably Hsp101 and the Hsp70 machinery (Fig. 5D, [71, 73]). On the other hand, function of HsfA2 in Arabidopsis was shown to depend on ROF1/FKBP62 and ROF2/FKBP65, which are prolyl cis/trans isomerase cochaperones of the Hsp90 machinery [121–123].

The discovery of an Hsf binding protein (HSBP1) as negative regulator of human Hsf1 [124] led to the identification of similar proteins also in plants. In maize an embryo lethal mutant *emp2* (empty pericarp 2) in fact results from non-functional EMP2, which is one of the two orthologous maize HSBPs. It can be speculated that tight control of Hsf function by EMP2 is mandatory for normal embryogenesis. Potential interaction partners of EMP2 were identified as HsfA2a, HsfA3, HsfA4d and HsfA5, whereas the second member, HSBP2 of maize, interacts with Hsfs A6a and A4a and cannot replace EMP2. No interaction with class B or C Hsfs was observed [125]. The Arabidopsis HSBP was also characterized as potential negative regulator of Hsf activities by interaction with Hsfs A1a, A1b and A2. Moreover, similar to maize, HSBP KO mutants of Arabidopsis are defective in seed development [126].

Important parts of the plant HS response and recovery at the transcriptional level are evidently regulated by a triad of functionally interacting Hsfs represented in tomato by HsfA1a, HsfA2 and HsfB1 (Fig. 5). Many facts about the function of these Hsfs including the chaperone interactions are known [35, 40, 69, 71, 72, 74]. In contrast to this, our knowledge about plant Hsf modifications are very fragmentary. Apparently, there is no comparable network as summarized for human Hsf1 in Fig. 4. In Arabidopsis, sumoylation of HsfA2 at Lys315 close to the C-terminus inhibits HsfA2 activity causing



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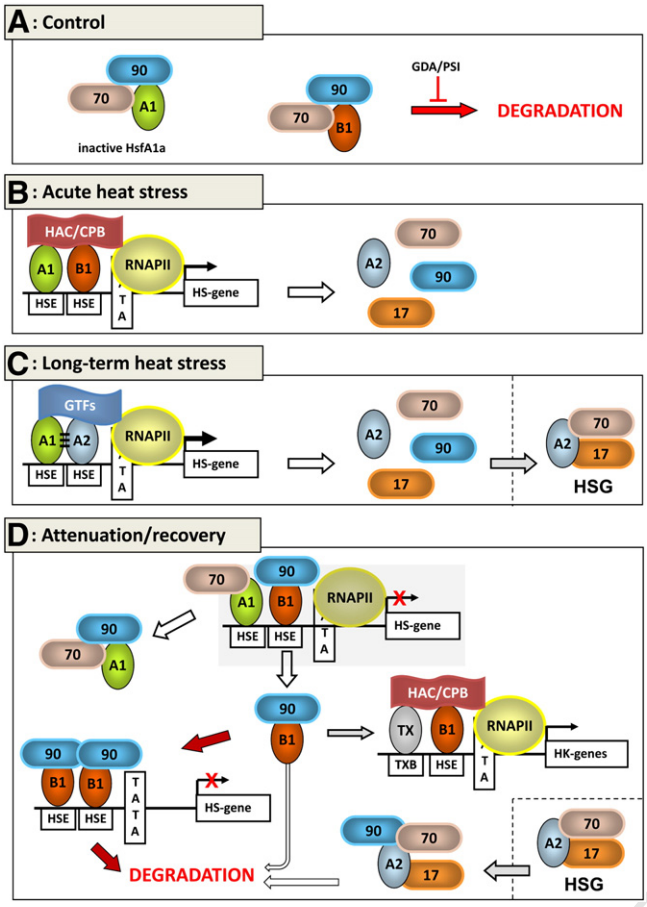


**Fig. 5.** The functional triad of HsfA1a, HsfA2 and HsfB1 in tomato and influence of chaperones on Hsf activity and stability (according to Hahn et al. [72]; modified). A: At control conditions, HsfA1a (A1) exists in an inactive state in complex with Hsp70/Hsp90 chaperones; the level of HsfB1 is kept low by degradation, which is inhibited in the presence of geldanamycine (GDA) or proteasome inhibitor (PSI). B: Upon HS induction HsfA1a is released from its complex with Hsp70/Hsp90; HsfA1a and HsfB1 cooperate in immediate activation of HS-inducible genes (Hsp70, Hsp90, Hsp17 and HsfA2). The A1/B1 transcription complex recruits HAC/CBP histone acetyl transferase to the transcription machinery. C: Long-term HS is connected with accumulation of high levels of chaperones and HsfA2. Together with HsfA1 HsfA2 forms the heterooligomeric superactivator complex (see Section 4.3). Part of the newly synthesized chaperones and HsfA2 are assembled into high molecular weight protein aggregates (heat stress granules, HSG). D: In the attenuation/recovery phase, transcription of HS genes is strongly decreased by interaction of HsfA1 with Hsp70 and HsfB1 with Hsp90; HsfA1a is inactivated; HsfB1 is degraded. In addition, HsfB1 interacts with house keeping transcription factors (TX) to restore house keeping gene expression. The release of proteins from HSG is mediated also by the Hsp90/Hsp70 chaperone machinery.

reduced Hsp synthesis and thermotolerance levels [127]. On the other hand, $Ca^{2+}$-dependent activation of MAP kinases under HS may result in phosphorylation of Hsfs and/or chaperones; but essential details remain to be clarified [128, 129].

### 5.3. The ER-based unfolded protein response of plants

The concept of accumulation and aggregation of denatured proteins in the cytoplasm as part of the stress sensing system (cytosolic protein response) leading to Hsf activation is broadly accepted (see Sections 5.1 and 5.2). But the same is true for the ER as second major cell compartment with protein folding and processing activities. The ER-based unfolded protein response (UPR) in eukaryotes is responsible for the adjustment of chaperone levels to the need of protein processing in this compartment [130, 131]. Signaling mechanisms in plants involve ER membrane-bound precursors of bZip transcription factors that undergo proteolytic cleavage and nuclear transport upon UPR [132–134]. Formation of another bZip factor results from stress-induced activation of the IRE1b splicing factor required for generation of the mature bZip60 mRNA [135]. Among the newly synthesized proteins are ER-specific chaperones like BiP and BAG as antiapoptotic protein [136].

### 5.4. Epigenetic effects of stress response

It is well known that modification of the chromatin state is an integral part of differential gene expression. Patterns of pre-activated or silenced genes marked by DNA methylation, association with non-coding RNAs and nucleosome modifications are stably propagated in a given cell lineage [137–144]. In all eukaryotes, modification patterns of histones rapidly change in the process of gene activation and transcription (histone code, [137]). As expected, this is also part of the plant stress response [145, 146]. Moreover, nucleosomes are also diversified by incorporation of histone variants, e.g. of the far spread H2A.Z, which is an important marker for the epigenetic memory of the chromatin state [147–149]. Kumar and Wigge [150] reported that H2A.Z containing nucleosomes are associated with heat and cold responsive genes of Arabidopsis and that H2A.Z is released upon stress induction. In keeping with this, plants with H2A.Z deficiency in their nucleosomes exhibit constitutively up-regulated HS genes. Another aspect of the HS response with respect to chromatin structure and epigenetic variations is the transient activation of repetitive elements or silenced gene clusters close to the centromeric regions [151] as well as the transient loss of epigenetic gene silencing [152].

## 6. Signaling mechanisms and stress integration

With the discovery that members of the Hsp families act as molecular chaperones involved in many aspects of protein homeostasis and cell signaling ([153]; see Introduction), the central concept of HS signaling always centered around the disruption of cytosolic protein homeostasis and depletion of the pool of free chaperones as the basis of Hsf activation (see Sections 5.1 and 5.2). But of course many other parts of cells such as membranes, cytoskeleton and metabolic networks sense temperature changes and create signals, e.g. $Ca^{2+}$, nitric oxide (NO), reactive oxygen species (ROS), metabolites, lipid signals, which all together contribute to the complexity of diverse temperature response systems. Thus, with respect to signal transduction, we face the problem of several if not many different "thermometers" [154–157].

Indeed, the Hsf controlled transcription of Hsp encoding genes is only a small part of the overall program of cellular HS response, which affects many house keeping and developmental functions of plants [2]. The same is true for mechanisms and components contributing to stress tolerance. Many other transcription factors [101, 145, 158–160], stress-induced proteins and metabolites, small non-coding RNAs as well as stress hormones such as ethylene (ETH), ABA, salicylic acid (SA) and jasmonic acid (JA) are integral parts of the highly complex response of plants as whole organisms in a stressful environment [5, 6, 91, 101, 161–165]. It is almost trivial to state that our focussed discussion of HS and Hsfs alone does not reflect the usual situation of plants in their natural surrounding when periods of high temperature are usually combined with water deficiency, nutrient deprivation, high light, and oxidative stress. The complexity of changes to such normal multistress situations is best illustrated by microarray analyses of gene expression patterns as compiled for Arabidopsis in the AtGenExpress initiative (see Section 4.9, [4, 166–168] or comprehensive analyses of metabolic changes [163, 169–171]. No doubt, improving knowledge about such stress-induced changes is essential to improve stress tolerance and productivity of cultural plants in a period of global climate changes [6, 9].

In the frame of this review on Hsf structure and function, we cannot go into all the exciting details of stress integration. But to illustrate the



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point, we would like to briefly mention few relevant examples indicating the tight connection of Hsf signaling with other parts of stress response.

- By screening of Arabidopsis mutants with defects in thermotolerance (*hot* mutants), Lee et al. [172] identified a mutant of S-nitrosoglutathione reductase. Evidently, NO homeostasis is essential for thermotolerance and development, and NO overproducing plants exhibit thermosensitive phenotypes. The original screening identified also mutants with defects in ABA and SA synthesis, ETH signaling, UV-sensitivity, and ROS signaling [173], and none of these mutants with defects in thermotolerance had reduced levels of Hsps. This indicates that, besides Hsps, many other components make significant contributions to the stress tolerance of plants.
- The HS response of the moss *Physcomitrella patens* coincides with activation of $Ca^{2+}$ channels and, at control temperatures, can be mimicked by perturbations of membrane fluidity [174]. On the other hand, $Ca^{2+}$ signaling is central to many other stress and hormonal response systems tightly connected with complex changes of protein phosphorylation patterns [175, 176]. In keeping with this, $Ca^{2+}$ binding protein calmodulin 3 (CaM3) in Arabidopsis is crucial for high levels of acquired thermotolerance [177], and $Ca^{2+}$-CaM3 acts downstream of NO signaling [178].
- The balance of ROS is important for survival and signaling not only in plants. Besides oxidative damage of proteins as part of cytosolic protein response, ROS have direct functions as HS signals [8, 179, 180]. ROS scavengers such as ascorbate impair HS-induced expression of chaperones.
- MBF1c (multiprotein bridging factor 1c) is a highly conserved transcription coactivator of eukaryotes. In Arabidopsis, it was shown to be involved into response to ETH as well as thermotolerance expression without affecting Hsp levels. MBF1c cooperates with WRKY39 transcription factor, which is well known from its role in SA and JA signaling pathways. The effects on thermotolerance levels evidently reflect the essential role of stress hormones and synthesis of stress metabolites such as trehalose, polyamines, proline and glycine betain for stress tolerance [160, 173, 180, 181]
- A HS-induced lipocalin represents a family of conserved proteins found in both prokaryotes and eukaryotes. Its importance for basal and acquired thermotolerance in Arabidopsis indicates that lipid peroxidation causes serious membrane damage and probably triggers the cell death response (apoptosis) under HS conditions [182].
- Expression of AtHsfs A6a and A6b is highly increased under salt and cold stress conditions (see Section 4.9, [106]). The special role of these two Hsfs for salt and drought stress response was confirmed by Yoshida et al. [183] using ABA signaling mutants with triple KO of the three known ABA dependent transcription factors AREB1, AREB2, and ABF3. All three were characterized as master regulator of the expression of drought responsive genes in the ABA-dependent signaling in response to water deficiency stress [183]. Microarray analysis of RNA expression patterns of Arabidopsis *areb1/areb2/abf3* triple mutants showed enhanced drought sensitivity and markedly impaired expression of drought responsive genes, among them Hsfs A6a and A6b. Unfortunately, studies about possible target genes of Hsfs A6a and A6b are lacking.

## 7. Concluding remarks

The striking multiplicity of Hsfs in flowering plants in the range of ~20-50 members and conserved patterns of structural and functional diversification between individual Hsfs correlates with the remarkable perfection in adaptation of land plants to growth and survival under a broad variety of stress situations. The basic function of class A Hsfs as activators of HS gene expression, as observed for Hsfs in all eukaryotic organisms, is complemented by additional roles in plant development and different stress responses. Although not analyzed in sufficient detail, members of class B Hsfs mostly have no activator function but rather act as repressors of gene expression. Nothing is known about the possible role of class C Hsfs with four or more representatives in monocots. Although studied only for few examples, chaperones (Hsp90, Hsp70 and Hsp17) are evidently involved into activity control, intracellular localization and stability of plant Hsfs. However, the whole complexity of interactions or cooperation between individual members of the family or of Hsfs with putative coactivators and corepressors respectively is just emerging.

## Acknowledgments

We acknowledge financial support of our research by grants from the German Research Council (DFG) to K.D.S. and L.N. T.B. is supported by the research funding initiative “LOEWE” of Hesse's Ministry of Higher Education, Research, and Arts. I.E. acknowledges support by a grant from the Wiener Wissenschafts und Technology Fond (WWTF) to Arndt von Haeseler. We thank Drs. Enrico Schleiff, Oliver Mirus and Markus Fauth for helpful discussions and critical comments during preparation of the manuscript and support in computer and art works. The authors greatly acknowledge Sebastian Schuster for setting up the Hsf database and for his help in the data analysis. We apologize to the authors whose work could not be cited due to space limitations.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbagrm.2011.10.002.

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Please cite this article as: K.-D. Scharf, et al., The plant heat stress transcription factor (Hsf) family: Structure, function and evolution, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagrm.2011.10.002

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Please cite this article as: K.-D. Scharf, et al., The plant heat stress transcription factor (Hsf) family: Structure, function and evolution, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagrm.2011.10.002

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Please cite this article as: K.-D. Scharf, et al., The plant heat stress transcription factor (Hsf) family: Structure, function and evolution, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagrm.2011.10.002

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Please cite this article as: K.-D. Scharf, et al., The plant heat stress transcription factor (Hsf) family: Structure, function and evolution, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbagrm.2011.10.002