Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors

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Compared to the overall multiplicity of more than 20 plant Hsfs, detailed analyses are mainly restricted to tomato and Arabidopsis and to three important representatives of the family (Hsfs A1, A2 and B1). The three Hsfs represent examples of striking functional diversification specialized for the three phases of the heat stress (hs) response (triggering, maintenance and recovery). This is best illustrated for the tomato Hsf system: (i) HsfA1a is the master regulator responsible for hs-induced gene expression including synthesis of HsfA2 and HsfB1. It is indispensible for the development of thermotolerance. (ii) Although functionally equivalent to HsfA1a, HsfA2 is exclusively found after hs induction and represents the dominant Hsf, the "working horse" of the hs response in plants subjected to repeated cycles of hs and recovery in a hot summer period. Tomato HsfA2 is tightly integrated into a network of interacting proteins (HsfA1a, Hsp17-CII, Hsp17-CI) influencing its activity and intracellular distribution. (iii) Because of structural peculiarities, HsfB1 acts as coregulator enhancing the activity of HsfA1a and/or HsfA2. But in addition, it cooperates with yet to be identified other transcription factors in maintaining and/or restoring housekeeping gene expression.

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1. Introduction

1.1 *Plant stress response*

During evolution, the origin of terrestrial plants (about 400 million years ago) required special adaptations to rapidly changing environmental conditions (Levitt 1980). Examples for these organismic adaptations are:

– The predominant role of the sporophyte in the life cycle of plants with the sensitive gametophyte being enclosed.

Keywords. Chaperones; heat stress transcription factors; plant heat stress response; thermotolerance

Abbreviations used: CBP, CREB binding protein; CS, cosuppression; HAT, histone acetyl transferase; hs, heat stress; HSE, heat stress promoter elements; HSG, heat stress granules; HSP, heat stress protein; NES, nuclear export signal; NLS, nuclear localization signal; OE, overexpression; WT, wild type.

– The organization of leaves as photosynthetic organs with the active cells inside, a protective outer layer of the epidermis and cuticle and, intimately connected with this organization, the necessary gas exchange proceeding through tightly controlled apertures (stomata).
– The formation of stress resistant dormant forms (seeds) for propagation and survival of unfavourable conditions.
– The development of a mechanically stabilized chormophyte allowing the generation of long-lived and very big plants with systems for long-distance nutrient and water transport.

But in addition to this, plants also became specialized to grow and propagate under extreme environmental conditions, e.g. under conditions of low or high temperatures, of high salt or heavy metal stress or of extreme water deficiency. As sessile organisms they developed remarkable capabilities to rapidly respond to multiple environmental changes from which they cannot escape. A network of interconnected cellular stress response systems is a prerequisite for plant survival and productivity (Nover *et al* 1989a; Cherry 1994; Brunold *et al* 1996).

1.2 *The molecular cell biology of the heat stress response*

Although stress responses of plants were studied experimentally since the middle of the 19th century (Sachs 1864; Müller-Thurgau 1980), a milestone for the analysis of cellular stress response systems was the pioneering work of the Italian developmental biologist F Ritossa working with the fruitfly *Drosophila melanogaster*. After a fortuitous increase of the temperature of the incubator, he observed striking changes of the puffing patterns, i.e. gene activity patterns of the polytene chromosomes in larval salivary glands (Ritossa 1962, 1996). Surprisingly enough, the same reprogramming of transcription was also observed after addition of chemical stressors like salicylate, 2,4-dinitrophenol and azide. About 10 years later A Tissieres and co-workers (Tissieres *et al* 1974) identified the newly formed heat stress proteins (Hsps). Soon, the rapidly developing field of heat stress biology included investigations in other eukaryotic organisms and bacteria. In fact, 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 (Nover *et al* 1989a; Nover 1991; Forreiter and Nover 1998; Scharf *et al* 1998a; Bharti and Nover 2002).

To present an overview of more than 40 years of molecular cell biology research in this field, we will use a hypothetical eukaryotic cell under stress (figure 1). The essential elements of this model can be summarized as follows (Wu 1995; Scharf *et al* 1998a; Morimoto 1998):

- Besides heat stress (hs), a large number of chemical stressors and various states of mammalian diseases cause activation of heat stress genes.
- Very likely, the stress sensing system in cells are deviations of protein homeostasis, i.e. of the equilibrium between new synthesis, folding, intracellular targeting, biological function and degradation of proteins. Proteins are shown in two states: (i) proteins in the native state (squares); and (ii) partially denatured proteins (stars) bound to chaperones.
- Heat stress proteins (Hsps) and constitutively expressed members of the conserved Hsp families are molecular chaperones essential for maintenance and/or restoration of protein homeostasis (see part 3 of this overview). 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.
- The transcription of Hsp encoding genes is controlled by regulatory proteins called heat stress transcription factors (Hsfs). They exist as inactive proteins mostly found in the cytoplasm. In figure 1, this is exemplified for the master regulator, HsfA1a, of the hs response in tomato (see below). Stress causes activation with oligomerization and, eventually, recompartmentation to the nucleus, where it binds to its target sequences (HSE) present in the promoter of hs genes (steps 1 and 2 of the Hsf cycle, on the left of figure 1).
- New synthesis of Hsps is assumed to replenish the pool of free chaperones. There is good evidence that in a kind of autorepression, some of the chaperones, e.g. Hsp17-CII, Hsp70, Hsp90 (Guo *et al* 2001; Kim and Schöffl 2002; Port *et al* 2004), are involved in the second part of the Hsf cycle leading to the restoration of the inactive state of hsfs in plants and animals (steps 3 to 5 of the Hsf cycle).

A peculiarity of plants is the unique complexity of the Hsf family with more than 20 members and the existence of hs-induced forms of Hsfs, which may play a major part in the modulation of transcription in the course of a long-term hs response. The complexity of the Hsf families is exemplified in figure 2 for three plants, i.e. for *Arabidopsis* and rice, whose genomes were sequenced (The *Arabidopsis* Genome Initiative 2000; Goff *et al* 2002; Yu *et al* 2002), as well as for tomato with the best studied Hsf system (Scharf *et al* 1990, 1998b; Treuter *et al* 1993; Boscheinen *et al* 1997; Bharti *et al* 2000, 2004; Döring *et al* 2000; Heerklotz *et al* 2001; Mishra *et al* 2002; Port *et al* 2004; see reviews by Nover *et al* 2001; Bharti and Nover 2002). Much information about the tomato Hsf system stems from the expressed sequence tag (EST) databases. References to the sequences used for the evolutionary tree in figure 2 can be found in table 1 and the following homepage (http://www.unifrankfurt.de/fb15/botanik/nover.html).

Compared to plants, the multiplicity of Hsfs is much smaller in other organisms. There are each one Hsf in yeast and *Drosophila* and three in vertebrates (Morimoto 1998; Nakai 1999; Nover *et al* 2001). Recently, a fourth, Hsf-like open reading frame (ORF) (HsfY) encoded on the Y chromosome was identified (Tessari *et al* 2004). Although the lack of HsfY has a clear phenotype (severe male infertility), its function as transcription factor needs to be investigated. The exceptional situation with the plant Hsf family prompts the question: What is the functional significance of 20 or more Hsfs? Do they represent a redundancy or do they fulfil specified functions either due to their structural peculiarities or to their divergent patterns of expression? Although our knowledge is far from complete, the few examples investigated so far with sufficient details indicate a remarkable diversification in the plant Hsf family.

2. Transcriptional control: Heat stress transcription factors as prototype of a eukaryotic activator protein

As indicated in figure 1, Hsfs are the central control proteins of the heat stress response. 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 promoter recognition are conserved throughout the eukaryotic kingdom. For the presentation in figure 3, we use three examples of *Arabidopsis* Hsfs with features typical for other plant Hsfs. In fact, many parts of the following explanations are also valid for Hsfs from yeast, *Drosophila* or mammals (Wu 1995; Nover *et al* 2001; Morimoto 1998; Bharti and Nover 2002):

(i) Close to the N-terminus the highly structured DNA-binding domain (DBD) is formed of a three-helical bundle

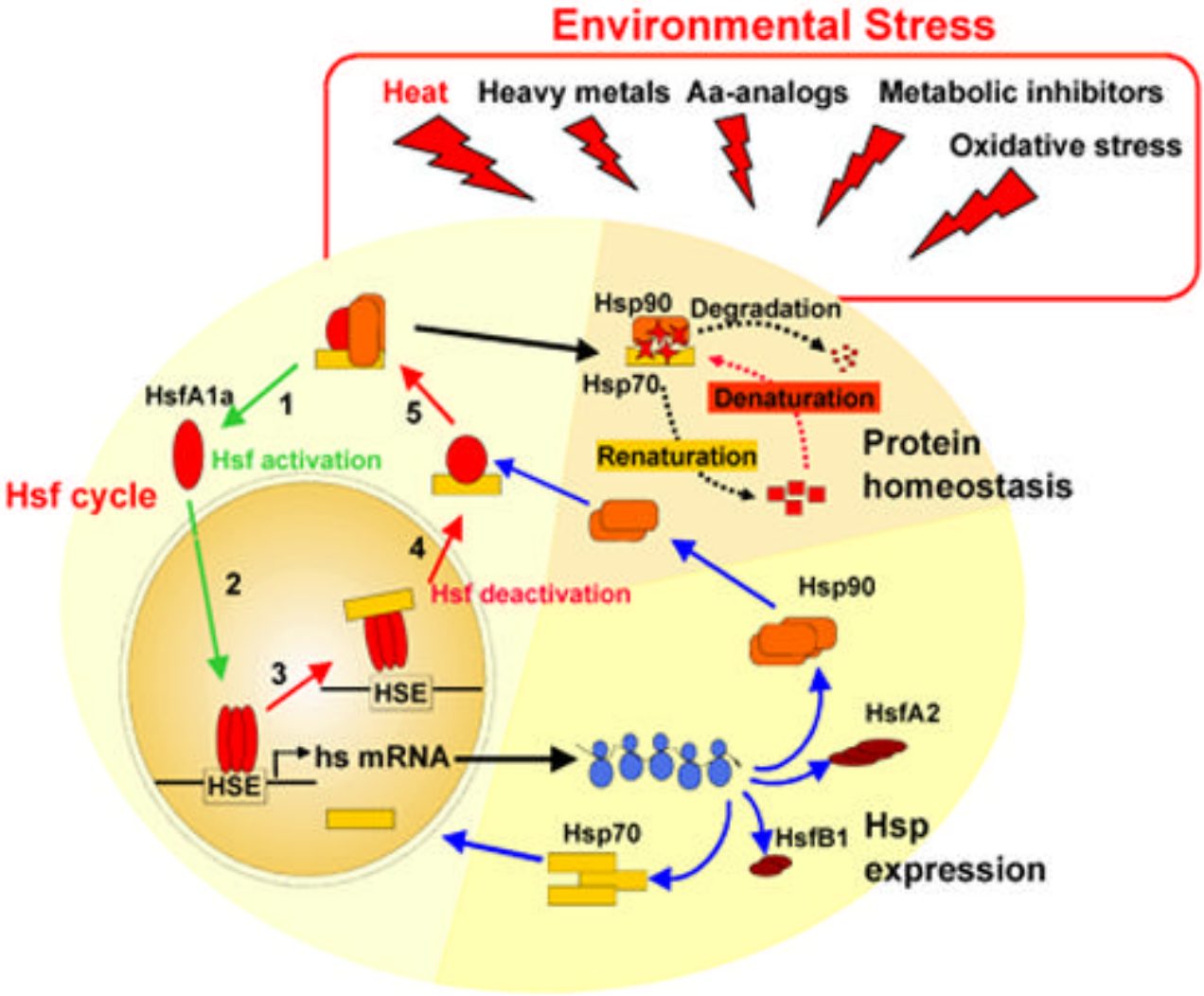

Figure 1. Heat stress transcription factors (Hsfs) as central regulators of the hs response. For explanations see text.

(H1, H2, H3) and a four-stranded antiparallel *b*-sheet (Damberger *et al* 1994; Harrison *et al* 1994; Vuister *et al* 1994; Schultheiss *et al* 1996). The hydrophobic core of this domain ensures the precise positioning of the central helix-turn-helix motif (H2-T-H3, Littlefield and Nelson 1999; Cicero *et al* 2001) required for specific recognition of the heat stress promoter elements (HSE). HSEs are formed of repetitive patterns of palindromic binding motifs (5′-AGAAnnTTCT-3′) upstream of the TATA box of hs-inducible genes (Pelham 1982; Nover 1991).

(ii) The oligomerization domain (HR-A/B region) is connected to the DNA-binding domain by a flexible linker of variable length (15 to 80 amino acid residues). A heptad pattern of hydrophobic amino acid residues in the HR-A/B

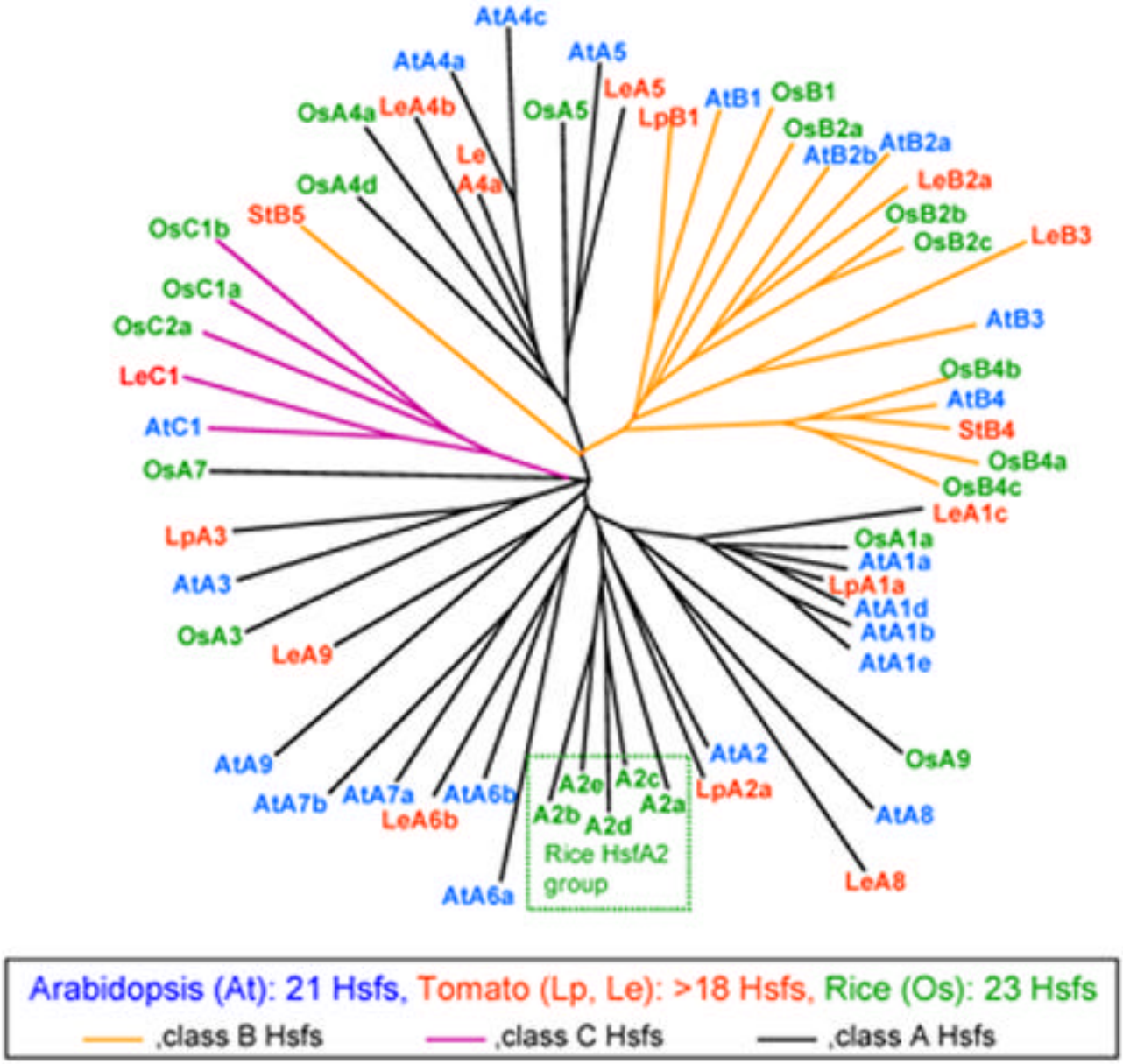

Figure 2. Phylogenetic tree of plant Hsfs. Using the Clustal software, the tree was generated on the basis of the amino acid sequences of the N-terminal domains of Hsfs including the DNA-binding domain, the HR-A/B region and parts of the linker between both (see figure 3). References to the corresponding clones of *Arabidopsis thaliana* (At), *Oryza sativa* (rice, Os) and *Lycopersicon esculentum* or *L. peruvianum* (tomato, Le/Lp) are given in table 1. Because the information on tomato Hsfs is incomplete, we included in two cases clone information from the closely related *Solanum tuberosum* (potato, St), assuming that HsfB4 (TC64536) and HsfB5 (TC65496) might also exist in tomato.

Table 1. Survey of ESTs (composite ESTs) encoding plant Hsfs (TC numbers represent composite ESTs created by the Institute of Genomic Research, TIGR).

	Arabidopsis thaliana (At)			*Lycopersicon esculentum* (Le)/ *Lycopersican peruvianum* (Lp)			*Oryza sativa japonica* (Os)		
Hsfs	Acc. No.	ORF (aa)	Intr. (nucl.)	Acc. No. (a)	ORF (aa)	Intr. (nucl.)	Acc. No. (b)	ORF (aa)	Intr. (nucl.)
HsfA1a	At4g17750	495	147	Le TC128701 Lp CAA47869	527	3477	AC120506(g) AK100430(c)	506	1386
HsfA1b	At5g16820	481	606	BE354387 (ex.1+intr.)					
HsfA1c				AW738023					
HsfA1d	At1g32330	482	1419	TC130996 (C-term.)					
HsfA1e	At3g02990	468	968						
HsfA2/2a	At2g26150	345	324	Le TC131560 LpCAA47870	351	488	AC092558(g) AK069579(c)	376	81
HsfA2b	–	–	–	–	–	–	AP003626(g) AK101824(c)	372	1323
HsfA2c	–	–	–	–	–	–	AC027658(g) AK072391(c)	358	1669
HsfA2d	–	–	–	–	–	–	AC105729(g) AK066844(c)	359	946
HsfA2e	–	–	–	–	–	–	AC092076(g) AK068660(c)	357	1199
HsfA3	At5g03720	412	532	Le TC125578 (ex.1+intr.) Le TC105313 (C-term.) Lp AF208544	508	727	AP004879(g) AK101934(c)	498	1298
HsfA4a	At4g18880	401	77	TC123111 (N-term.)					
HsfA4b				TC107140 (partial)	388		AP003076(g) AK109856(c)	444	582
HsfA4c	At5g45710	345	93(c)						
HsfA4d				AW034402 (N-term)			AC111015(g) AK100412(c) AP004086(g)	459	237(c)
HsfA5	At4g13980 (d)	466	328	TC125429	478		AP004999(g) AK072210(c) AK065643(c)	475	1463
HsfA6a	At5g43840 (d)	282	80						
HsfA6b	At3g22830	406	694	TC125038 TC125037 (defective)	335	922	AP003308(g) AK064271(c)	402	1406
HsfA7a	At3g51910	272	507						
HsfA7b	At3g63350	282	496						
HsfA8	At1g67970	374	345	TC120227 (C-term)					
HsfA9	At5g54070	331	79	TC131356 (N-term) AI487342 (C-term)			AC107226(g) AK072571(c)	410	1231
HsfB1	At4g36990	284	193	Le TC119447 Lp CAA39034	301	1645	AP006057(g) AK101182(c) AK061433(c)	302	5810
HsfB2a	At5g62020	299	83	TC109214 AW220758 (exon1+intr.)	338		AL663003(g)	305	102
HsfB2b	At4g11660	377	89				AP004704(g) AK101700(c)	390	101
HsfB2c							AP005681(g) AK106525(c)	454	120
HsfB3/B3a	At2g41690	244	108	AI898398(N-term.) BQ514655(C-term.)					
HsfB3b				BI209938					
HsfB4/4a	At1g46264	348	233				AP004693(g)	380	93
HsfB4b							AP004671(g) AK063952(c) AP005292(g)	310	1665
HsfB4c							AP005655(g)	394	88
HsfB4d							AC125784(g)	305	145
HsfC1/1a	At3g24520	330	84	TC127986			NP304058(g) AK069479(c)	339	79
HsfC1b							AP003560(g) AK066316(c)	412	108
HsfC2a							AP004070(g) AK106488(c)	298	86
HsfC2b							AP003682(g)	trunc.	1128

(a) Some ESTs contain intron sequences, mostly ESTs encoding the 5′ exon 1, e.g. of tomato Hsfs A1b, A3, B2a.
(b) For identification of rice Hsf encoding clones the genomic clones or contigues (g) and the full length cDNA clones (c) are given.
(c) The genes encoding *Arabidopsis* HsfA4c and the rice HsfA4d contain a second intron in the 5′UTR of 406 nucl. (At) and 243 nucl. (Os) respectively.
(d) Compared to the officially annotated clones in the MIPS data base, we corrected the *Arabidopsis* clone for HsfA5 by deleting a long additional exon added erronously to the 5′ end and the clone for HsfA6a by deleting the computergenerated intron in the CTD.

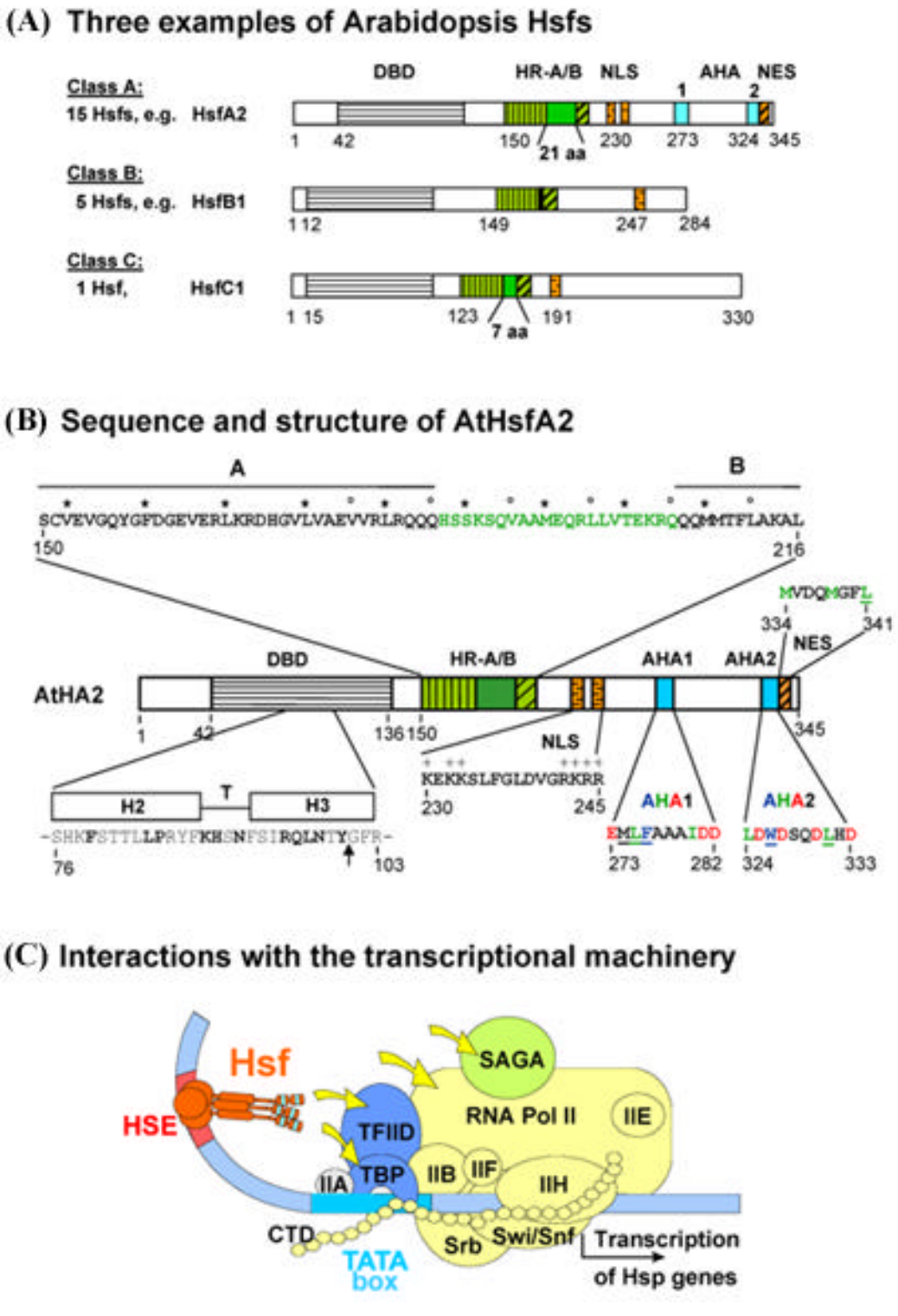

Figure 3. Basic structure of Hsfs and putative interactions with components of the transcription machinery. (**A**, **B**) Three types of *Arabidopsis* Hsfs representing class A, class B and class C Hsfs with their functional domains. Sequence details of the functional domains are given for HsfA2 in (**B**). For details see text. (**C**) Model of class A Hsf interacting through its AHA motifs with components of the basal transcription machinery (yellow arrows) composed of RNA polymerase II, transcription factors TFIIA, IIB, IID, IIE, IIF and IIH as well as TBP and the coactivator complexes Srb, Swi/Snf and SAGA.

region leads to the formation of a coiled-coil structure characteristic of proteins with leucine zipper type interaction domains (Peteranderl *et al* 1999). In plants, there are three classes of Hsfs (classes A, B and C), which are discriminated by peculiarities of their flexible linkers and by their HR-A/B regions (Nover *et al* 2001; Kotak *et al* 2004, see figure 3). The HR-A/B region of class B Hsfs is compact and similar to all non-plant Hsf, whereas all class A and class C Hsfs have an extended HR-A/B regions due to an insertion of 21 (class A) and 7 (class C) amino acid residues between the A and B parts (see arrows in figure 3A and sequence details given in figure 3B).

(iii) The nuclear localization signal (NLS) of Hsfs is mostly formed by bipartite clusters of basic amino acid residues (Lyck *et al* 1997). These positively charged recognition motifs (see details in figure 3B) help in the assembly of a nuclear import complex built of the target protein, e.g. HsfA2, the NLS receptor (importin *a*) and two other subunits, importin *b* and Ran(GTP) (Mattaj and Englmeier 1998; Görlich and Kutay 1999).

(iv) In fact, the intracellular distribution of Hsfs changes dynamically between nucleus and cytoplasm, and this phenomenon was shown to depend on the balance of nuclear import and export. A hydrophobic, frequently leucine-rich nuclear export signal (NES) at the C-terminus of many Hsfs (Heerklotz *et al* 2001) is required for the receptor-mediated export in complex with the NES receptor exportin-*a* (Görlich and Kutay 1999). Together with the activator modules (AHA motifs), the NES serves as part of a type-specific signature region in the C-terminus of class A Hsfs of plants (Kotak *et al* 2004).

(v) The function of Hsfs as transcription activators evidently resides in short activator peptide motifs (AHA motifs) in their C-terminal domains characterized by aromatic (W, F, Y), large hydrophobic (L, I, V) and acidic (E, D) amino acid residues (Treuter *et al* 1993; Döring *et al* 2000; Kotak *et al* 2004; see examples given in figure 3B). Similar AHA motifs were found and functionally characterized in the centre of many other transcription factors of yeast and mammals, e.g. VP16, RelA, Sp1, Fos, Jun, Gal4, Gcn4 as well as the steroid and retinoic acid receptors (see summary and references in Döring *et al* 2000; Kotak *et al* 2004). Most likely, they represent the essential sites of contacts with subunits of the basal transcription complex (figure 3C). Tjian and Maniatis (1994) 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 target proteins. 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* (Regier *et al* 1993; Schmitz *et al* 1994; Lin *et al* 1994; Barlev *et al* 1995; Melcher and Johnston 1995; Jackson *et al* 1996; Kotak *et al* 2004).

3. Heat stress proteins as molecular chaperones

As outlined in § 1·2, all organisms respond to supraoptimal temperatures by synthesizing a specific set of Hsp. They are needed to protect cells from heat damage, and they assist in normalization of functions during recovery (reviewed by Nover 1991; Nover and Scharf 1997; Bukau and Horwich 1998; Feder and Hofmann 1999). Heat stress proteins can be assigned to families of proteins conserved among bacteria, plants and animals (table 2). During the last decade more and more details of the biochemical function of Hsps have emerged. As molecular chaperones they assist in folding, intracellular distribution, assembly and degradation of proteins, mainly by stabilizing partially unfolded states (Ellis 2000; Forreiter and Nover

Table 2. Survey of major chaperones/chaperone systems and their function in eukaryotes.

Chaperones/chaperone systems (a)	Function in eukaryotes
Hsp101 (ClpA/B/X)	ATP-dependent dissociation of protein aggregates
Hsp90 (HtpG)	Coregulator of signal transduction complexes
Hsp70/Hsp40 (DnaK/J)	Primary stabilization of newly formed proteins, ATP-dependent binding and release (see figure 4)
Hsp60/Hsp10 (GroEL/S)	Specialized folding machinery, ATP-dependent
Hsp20	Form high molecular weight oligomeric complexes which serve as matrix for stabilization of unfolded proteins; Hsp70/40 and/or Hsp101 needed for release
Folding catalysts	Disulfide isomerases, Peptidyl prolyl-*cis*/*trans* isomerases (cyclophilins, FKBPs)

(a) Names given in parentheses define prokaryotic orthologs of the chaperones.

1998; Hartl and Hayer-Hartl 2002; Haslbeck 2002; Young *et al* 2003; Soll and Schleiff 2004; Wegele *et al* 2004). Chaperones do not contain specific information for correct folding, but rather prevent unproductive interactions (aggregation) between non-native proteins. This type of molecular chaperones can be distinguished from other heat-induced proteins acting as direct folding catalysts like peptidyl-prolyl isomerases or protein disulfide isomerases (Schmid 1995; Gething 1997; Gothel and Marhiel 1999; He *et al* 2004; Tu and Weissman 2004; Kadokura *et al* 2003).

Four major aspects in the life cycle of proteins invoke chaperone activities (Gething and Sambrook 1992; Gething 1997, see figure 4): (i) they ensure that nascent polypeptides emerging from the ribosome are kept in a folding competent state until the whole sequence information is available. (ii) Since fully folded proteins cannot be translocated through membranes, chaperones are needed to maintain or create a partially unfolded form of proteins destined for the import into mitochondria or plastids (Braun and Schmitz 1999; Neupert and Brunner 2002; Rehling *et al* 2003; Wiedemann *et al* 2004; Soll and Schleiff 2004). (iii) They stabilize damaged proteins generated as a result of chemical or physical stress and thus facilitate renaturation and/or degradation in the recovery period. (iv) They assist and control assembly and disassembly of multiprotein complexes (Lorimer 2001; Kim *et al* 2002).

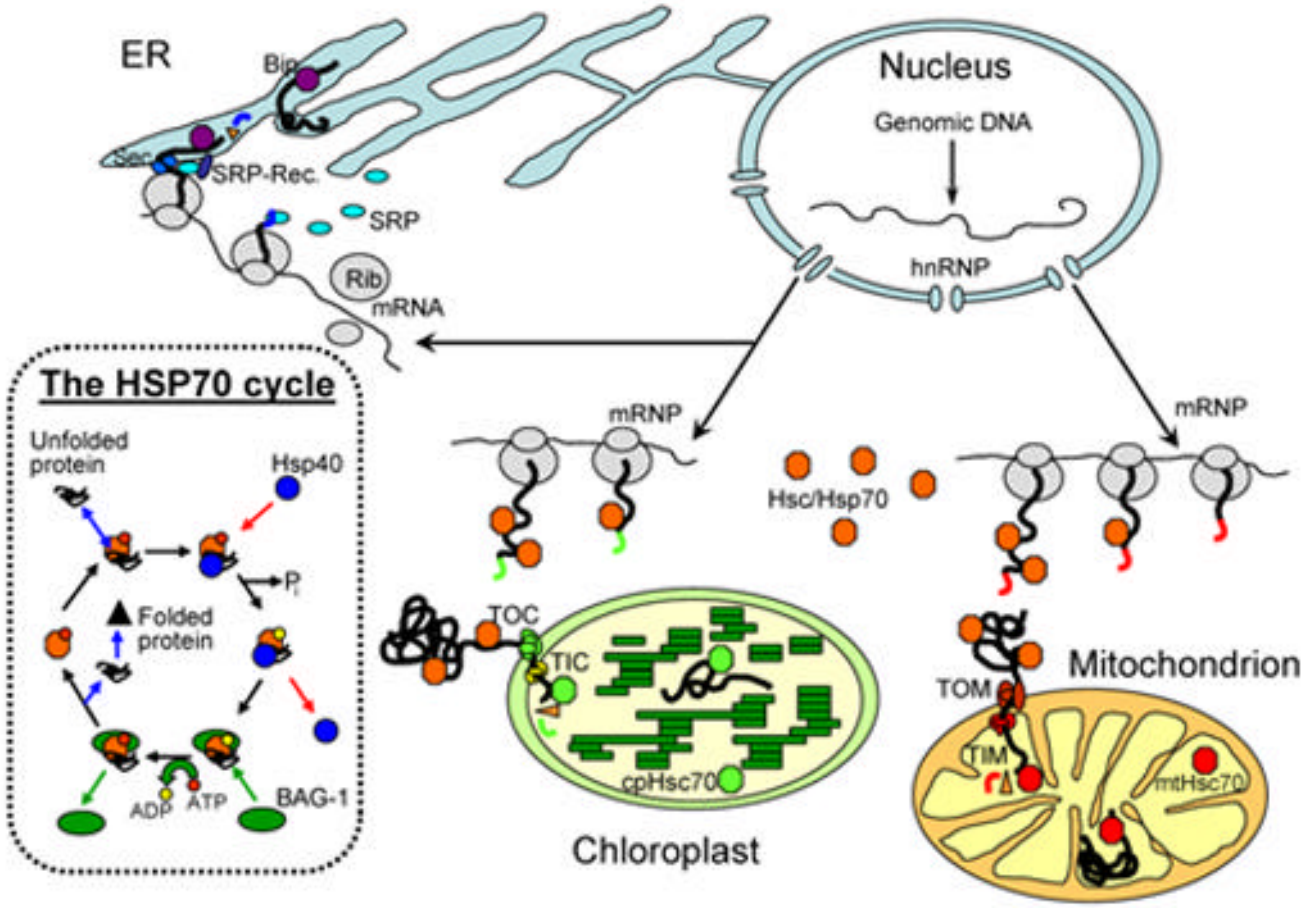

Figure 4. Cooperation in the chaperone network exemplified for the role of Hsp70 chaperone machines in the cytoplasm, ER and organelles for protein import. Nascent protein chains emerging from the ribosomes are bound by cytosolic forms of Hsc/Hsp70 (orange). Depending on the nature of the N-terminal signal sequence, proteins are imported post-translationally into the chloroplasts or mitochondria. In both cases a protein import chanel is formed of an outer membrane (TOC/TOM) and inner membrane (TIC/TIM) multiprotein complex. Partially unfolded proteins are delivered to the pore complex and imported in an ATP consuming process involving the organellar Hsc70. For import into the ER, translation is arrested by the SRP particle as soon as the ER-specific signal sequence emerges from the ribosome. Translation continues only after SRP contact with the Sec complex in the ER membrane allows cotranslational insertion of the protein.

Insert with the Hsp70 cycle: The Hsc/Hsp70 ATP-binding chaperones usually cooperate with two other proteins, a DnaJ-type subunit (Hsp40) for substrate recognition and control of ATPase function and a nucleotide exchange factor. This is exemplified for the cytosolic Hsc/Hsp70 cycle.

Many data collected during the last few years indicate that members of different Hsp families act together in so-called chaperone machines (Bukau and Horwich 1998; Walter and Buchner 2002; Young *et al* 2003; Wegele *et al* 2004), and different chaperone complexes may interact to generate a network for protein maturation, assembly and targeting (Frydman 2001; Johnson and Craig 1997; Forreiter and Nover 1998; Lee and Vierling 2000). Although many proteins are potential substrates for chaperone machines, e.g. after stress-induced protein damage, most of them (about 80%) fold in a chaperone-independent manner under normal conditions (Netzer and Hartl 1998). It is assumed that proteins at the surface of the ribosomes may help to stabilize nascent polypeptide chains (Frydman 2001; Hartl and Hayer-Hartl 2002).

4. Functional diversification of plant Hsfs

4.1 *Identification of HsfA1a as master regulator of induced thermotolerance in tomato*

As a first example for functional diversification we want to describe the case of tomato HsfA1a which is the master regulator for hs response and in this function cannot be replaced by any other Hsfs, at least not *in planta* (Mishra *et al* 2002). The evidence for this stems from two different transgenic tomato lines with altered expression of HsfA1a (figure 5) generated by incorporation of *HsfA1* transgene cassettes: (i) an over-expression (OE) line, containing a single *HsfA1a* transgene cassette (figure 5A), that has a 10- to 15-fold increased level of the HsfA1a (figure 5D)

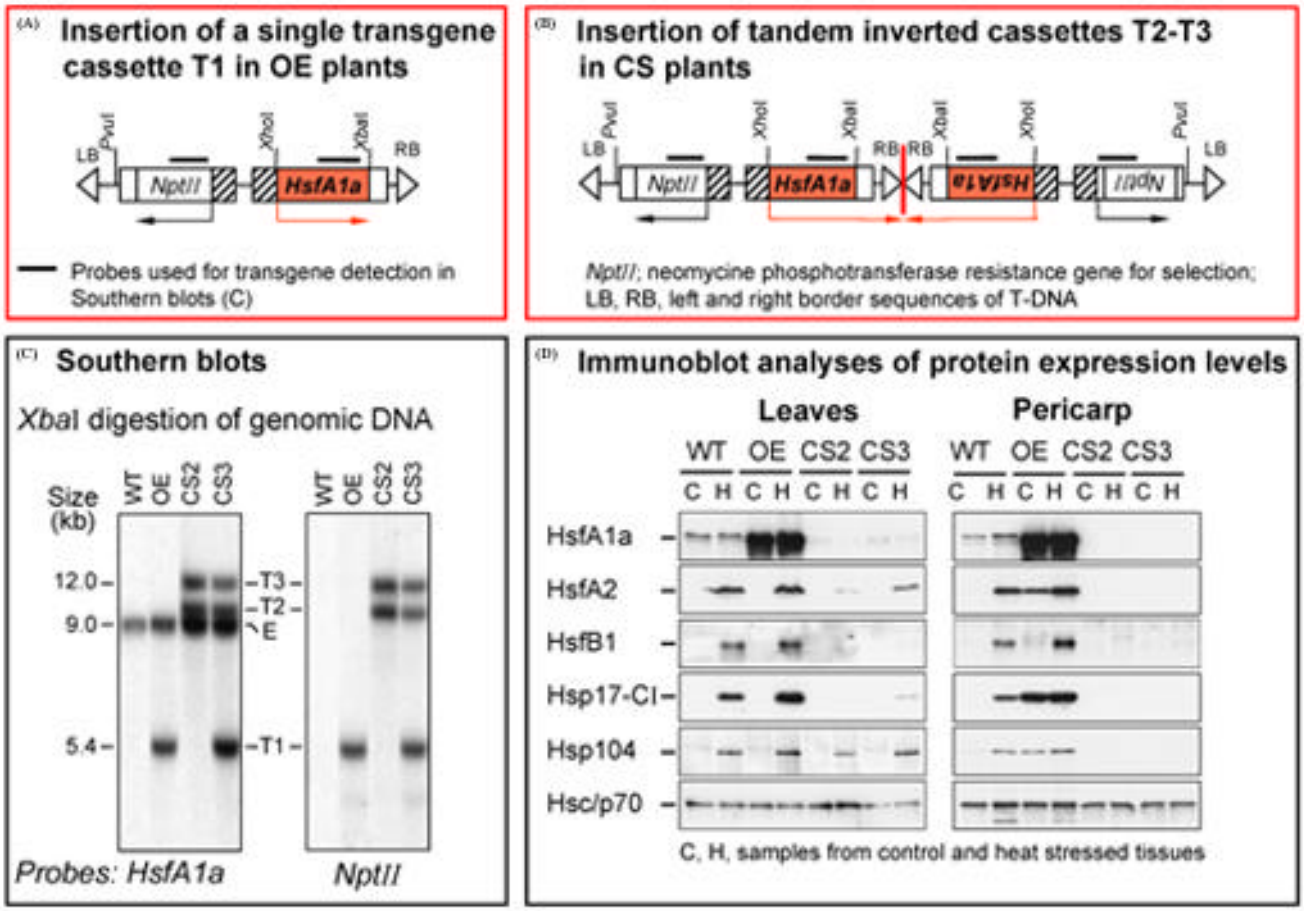

Figure 5. Characterization of transgenic tomato plants with altered expression of the master regulator HsfA1a (from Mishra *et al* 2002, modified). (**A**, **B**) Transgenic plants were generated by Agrobacterium-mediated gene transfer. Two modes of insertion of the transgene unit composed of the expression cassettes for the neomycin phosphotransferase (NptII) resistance marker and for HsfA1a are shown. Both cassettes are inserted between the left and right borders of the T-DNA (LB, RB). (**C**) Southern blot analysis of the transgene situation in WT plants with the endogenous gene only, in HsfA1a OE plants harbouring an additional T1 transgene (see **A**) and in the CS lines with the T2-T3 tandem inverted repeat (see **B**). CS3 plants contain both the T1 and the T2-T3 transgenes in their genome. E marks the position of the endogene. (**D**) Analysis of protein expression levels in control (C) and heat stressed (H) leaves and pericarp using specific antibodies against the indicated Hsfs and Hsps.

and (ii) a co-suppression (CS) line with two cassettes of transgene inserted as a tandem inverted repeat (figure 5B). This leads to the synthesis of double stranded small interfering RNA (siRNA) and post-transcriptional gene silencing (PTGS) of both the transgene and the endogenous gene of HsfA1a. As a consequence, no HsfA1a is detectable in leaves and pericarp of CS lines (figure 5D).

What are the biological consequences of the marked changes in HsfA1a expression levels between wild type and transgenic lines (figure 5D)? Under normal growth conditions, all three lines showed no obvious phenotype (figure 6A). But exposure to a mild hs treatment (1 h at 45°C ambient temperature) documented that the CS plants, are unable to acquire thermotolerance. They died soon after the stress treatment (figure 6B), whereas wild type (WT) and OE plants were not visibly affected. However, exposure to a severe heat stress (1 h at 45°C followed by 1 h at 51°C ambient temperature) was also lethal to the WT plants, but OE plants survived documenting their higher level of adaptive capacity (figure 6C). In correspondence with the phenotypic effects (figure 6), the expression levels of hs-inducible chaperones were markedly changed in correlation with the level of HsfA1a in the three genetic lines (figure 5D). Although the RNAi effect is not equally strong, the reduction of the chaperone expression levels is clearly visible in both leaves and pericarp. Unfortunately, we could not discriminate the constitutively expressed and hs induced forms of the Hsp70/Hsc70 complex. This results from the cross reactivity of the antiserum used for detection. However using RT-PCR, we could clearly show that the level of expression of Hsp70 mRNA was strongly reduced in the CS plants (data not shown in figure 6).

The extraordinary heat sensitivity of CS plants was also visualized by studying the developmental program of fruit ripening (Alexander and Grieson 2002; Bramley 2002). To this aim, mature green fruits of all three lines were harvested (samples 1) and subjected to a 2 days heat treatment at 42°C before storage for ripening in the dark at 25°C for 21 days (see pictograph in figure 6D). As indicated by the characteristic lycopene synthesis, normal ripening had occurred in fruits from WT and OE plants

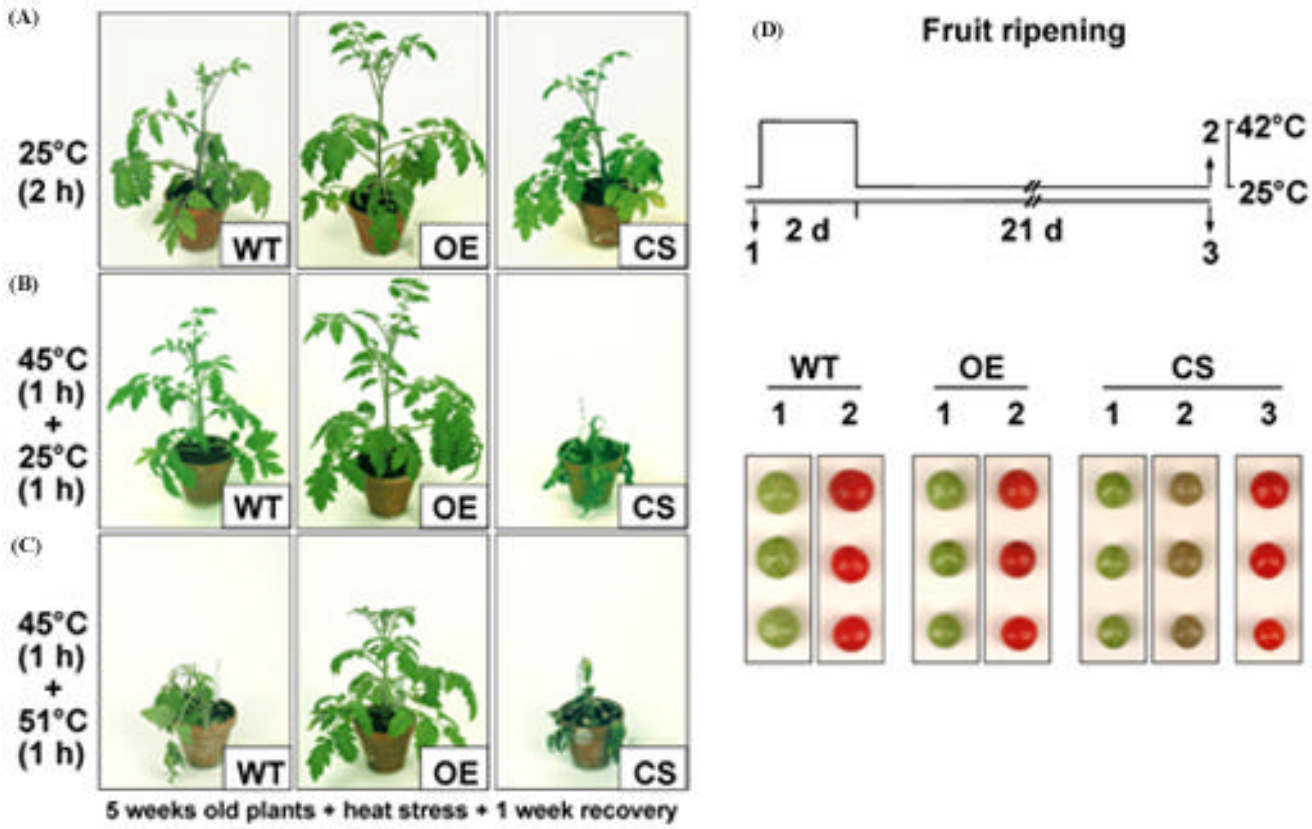

Figure 6. Thermotolerance in wild type and transgenic tomato plants with altered expression of HsfA1a (from Mishra *et al* 2002, modified). Whole plants (**A–C**) and fruits (**D**) of the plants described in figure 5 were subjected to the indicated heat stress and recovery treatments. Time points of harvesting of fruit samples 1, 2 and 3 are indiated at the pictograph. For further details see text.

(samples 2). However, fruits from CS plants were damaged, their pericarp was brown and no lycopene synthesis was observed. As a control, CS fruits, kept in the dark without the prior heat treatment, showed normal ripening behaviour (sample 3, figure 6D).

Although from the phylogenetic analysis, the Hsf families of tomato and *Arabidopsis* seem to be similar in complexity and basic composition (see figures 2 and 3), so far no similar master regulator Hsf has been identified in *Arabidopsis*. The group of F Schöffl showed that neither knock-out of AtHsfA1a nor of AtHsfA1b nor of both together (Lohmann *et al* 2004) had marked effects on the heat stress response and the long-term thermotolerance of *Arabidopsis*, which would be comparable to the severe thermotolerance defects observed in the tomato CS lines (Mishra *et al* 2002). From the point of view of evolutionary adaptation to survive under stressful conditions, the situation with a single master regulator in tomato appears very risky, whereas *Arabidopsis* seems to provide more flexibility. However, more comparative analyses of Hsf function in both plants are required to justify such a conclusion.

4.2 *Synergistic activation of gene expression by tomato Hsfs A1a and B1 includes recruitment of HAC1/CBP as coregulator*

In contrast with the class A heat stress transcription factors (Hsfs) of plants, a considerable number of Hsfs assigned to classes B and C have evidently no function as transcription activators if tested alone in appropriate reporter assays. In particular, they are lacking the characteristic contact motifs (AHA motifs) to interact with components of the basal transcription machinery (figure 3C). However, experimental evidence indicates that tomato HsfB1 represents a novel type of coactivator cooperating with class A Hsfs, e.g. with tomato HsfA1a (Bharti *et al* 2004). Provided the appropriate promoter architecture for adjacent positioning of the two Hsfs, they assemble into an enhanceosome-like complex (Carey 1998; Näär *et al* 2001) resulting in strong synergistic activation of reporter gene expression. Moreover, HsfB1 also cooperates in a similar manner with other activator proteins, e.g. with the bZip activator proteins binding to the cauliflower mosaic virus 35S promoter (TGA2; Lam 1994; Niggeweg *et al* 2000) or with yet unidentified activators controlling housekeeping gene expression. By these effects, HsfB1 may help to maintain and/or restore expression of certain viral or housekeeping genes during ongoing heat stress. The models in figure 7 reflect three possibilities of gene expression control by HsfB1 as derived from the experimental data of Bharti *et al* (2004). The special role of HsfB1 underlines a typical trait of the plant stress response systems. The organism needs to be pre-programmed for the recovery period, i.e. for the rapid resumption of housekeeping and developmental gene expression. The efficiency of switching between the two modes of gene expression in the stress and recovery period maybe crucial for plants adapted to grow under rapidly changing environmental conditions.

The coactivator function of tomato HsfB1 depends on a histone-like motif in its C-terminal domain with an indispensable lysine residue (K) in the centre (GRGKMMK). This motif is required for recruitment of the plant CREB binding protein (CBP) ortholog HAC1. HsfA1, HsfB1 and HAC1/CBP form ternary complexes *in vitro* and *in vivo* with markedly enhanced efficiency in promoter recognition (Bharti *et al* 2004). Mammalian CBP is a 300 kDa global coactivator with histone acetyl transferase (HAT) activity. As a type of scaffold protein, it interacts with many transcription factors either bound to its N-terminal or C-terminal domains (Nakashima *et al* 1999; Stern and Berger 2000; Bannister and Kouzarides 1996; Chan and LaThangue 2001; Yuan and Giordano 2002). Five CBP-like proteins were identified in *Arabidopsis* (HAC1 to HAC5; Bordoli *et al* 2001; Yuan and Giordano 2002). Despite a considerable extent of sequence variation, all five contain the most conserved central parts of mammalian CBP with the three Zn finger (C/H1 to C/H3) and the HAT domains (see blocK-Diagrams in figure 7D).

The specificity of the responses with HsfB1 is exemplified for a reporter assay in tobacco protoplasts using four different *Arabidopsis* promoters hooked up to a Myc-tagged Hsp17·6A-CI gene as reporter (figure 8A). Protoplasts were transformed with combinations of the reporter plasmid with the empty activator plasmid as control (sample 1), with HsfA1a expression plasmid (sample 2), with HsfB1 expression plasmid (sample 4) and with both together (sample 3). The first three promoters (figure 8A, a to c) representing house-keeping genes respond strongly to the presence of HsfB1 (samples 4) but not or much weaker to HsfA1a (samples 2). In contrast to this, the hs-induced promoter derived from the Hsp70 gene (figure 8A, d) was completely dependent on the presence of HsfA1a (sample 2), and its activity was optimum in the presence of HsfA1a and HsfB1 (sample 3). The intriguing differences in the response between reporters containing promoter fragments derived from the house-keeping Hsc70 (reporter c) and the hs-induced Hsp70 genes (reporter d) reflect the situations for cooperation of HsfB1 envisaged in figure 7C and A respectively.

4.3 *The dominant role of tomato HsfA2 and function of Hsp17-CII as corepressor*

Due to the high activator potential and its continued accumulation during repeated cycles of heat stress and re-

covery, HsfA2 becomes a dominant Hsf in tomato. Its activity is controlled by a network of proteins influencing its solubility, intracellular localization and activator function (figure 9). By interaction with the C-terminal domain of HsfA2, Hsp17·4-CII acts as specific corepressor (Port *et al* 2004). Eventually, both proteins together form large cytosolic aggregates, which can be solubilized either in presence of class CI sHsps or by hetero-oligomerization with HsfA1a leading to nuclear retention of HsfA2. Although different in its physicochemical properties, the situation with *Arabidopsis* HsfA2 is principally similar. It represents also a highly expressed and hs-inducible Hsf, and its activity in corresponding reporter assays was repressed in presence of AtHsp17·7-CII.

But tomato HsfA2 has further remarkable features. Due to a strong C-terminal NES, it does not localize in the nucleus unless co-expressed with the constitutively present master regulator HsfA1a. Evidently, HsfA1a is not only required for the hs-dependent expression of HsfA2, but also as coactivator and nuclear retention factor by formation of HsfA1a/HsfA2 hetero-oligomers (Scharf *et al* 1998; Heerklotz *et al* 2001; Port *et al* 2004).

Finally, the network of protein interactions influencing the function and intracellular distribution of HsfA2 has a hs-specific aspect. In the course of a heat stress response, the ongoing accumulation of HsfA2 and other hs-inducible proteins results in a unique storage form of the transcription factor in cytoplasmic multi-chaperone complexes

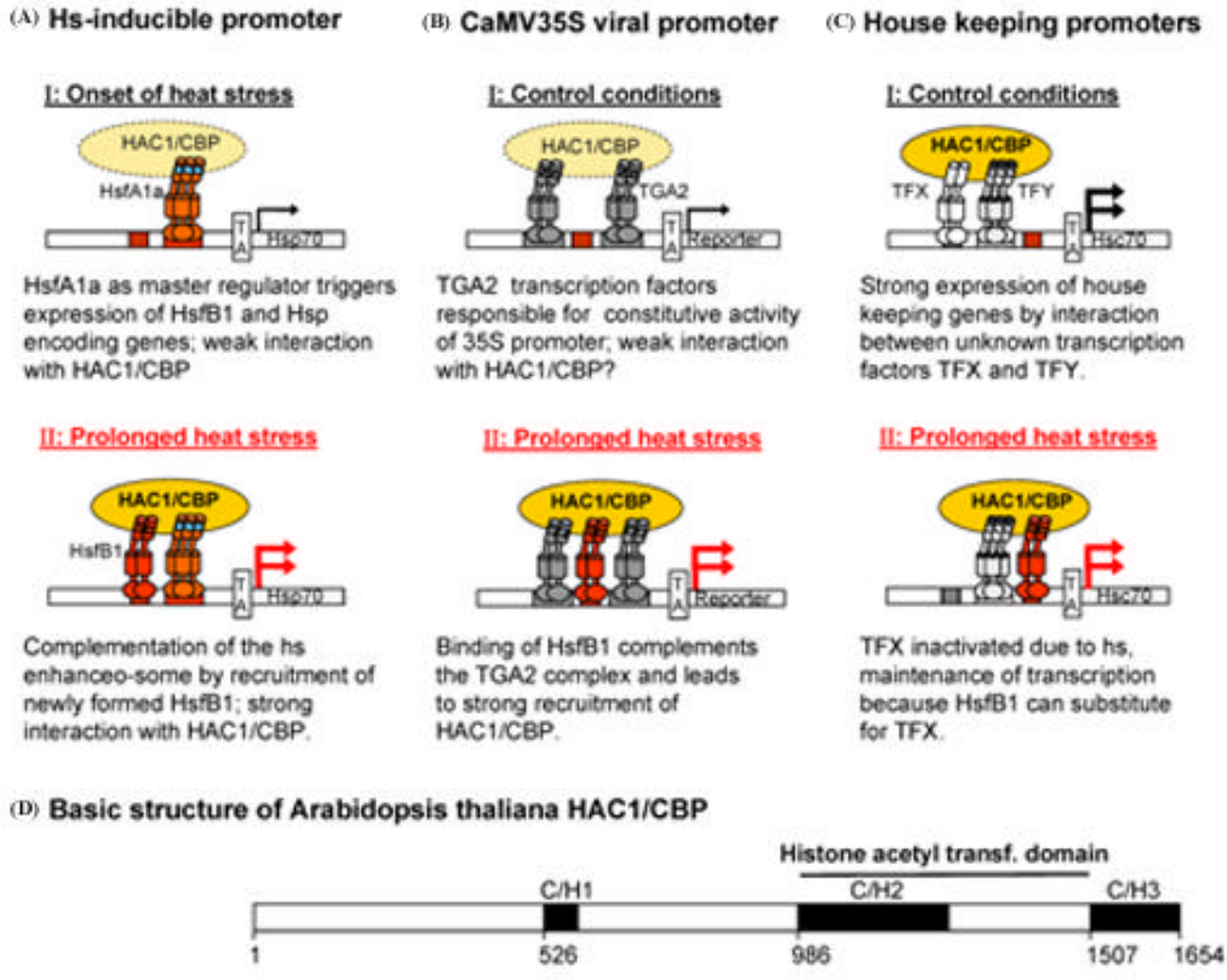

Figure 7. Model of HsfB1 as coactivator of HsfA1a (**A**) or functionally equivalent activators controlling viral (**B**) or house-keeping (**C**) gene expression (from Bharti *et al* 2004, modified). The basic structure of *Arabidopisis thaliana* HAC1/CBP is shown in (**D**). Cooperation of HsfB1 with acidic activators in a type of enhanceosome (Carey 1998) leads to strong co-recruitment of HAC1/CBP and enhanced stimulation of transcription (double arrows). For further explanations see text.

composed of the 40 nm heat stress granules (HSG, Nover *et al* 1989b; Scharf *et al* 1998b). No other Hsf so far identified in tomato cells (HsfA1, HsfA3, HsfB1) was found in the HSG complexes (Scharf *et al* 1998; Bharti *et al* 2000), which are mainly formed of the cytosolic small Hsp (sHsp) classes CI and CII and Hsp70. Dissociation of HSG complexes in the recovery with liberation of HsfA2 needs cooperation with the ATP-dependent Hsp70 and/or Hsp101 chaperone machines (K-D Scharf, unpublished).

5. Conclusions

Compared to the overall multiplicity of plant Hsfs, our analyses so far are mainly restricted to a single plant (tomato) and to three important representatives of the family (Hsfs A1a, A2 and B1). Besides basic similarities, the three Hsfs represent examples of striking functional diversification putting them into very different positions within the hs response.

(i) HsfA1a is the master regulator responsible for hs-induced gene expression including synthesis of HsfA2 and HsfB1. It is indispensable for the development of thermotolerance.

(ii) HsfA2 is functionally equivalent to HsfA1a, but it is exclusively found after hs induction. Probably, it represents the dominant Hsf, the "working horse" of the hs response in tomatoes subjected to repeated cycles of hs and recovery as experienced in a hot summer period. HsfA2 is tightly integrated into a network of interacting proteins (HsfA1a, Hsp17-CII, Hsp17-CI) influencing its activity and intracellular distribution.

(iii) Finally, because of its structural peculiarities, HsfB1 acts as coregulator enhancing the activity of HsfA1a and/or HsfA2. But in addition, it cooperates with yet to be identified other transcription factors in maintaining and/or restoring house-keeping gene expression.

As mentioned before, the situation in *Arabidopsis* seems to be different in several aspects. A single Hsf as master regulator could not be identified (Lohmann *et al* 2004). In addition, *Arabidopsis* HsfB1 is not comparable to its tomato counterpart. The –GRGKMMK-motif, essential for recruitment of HAC1/CBP, is defective, and AtHsfB1 was

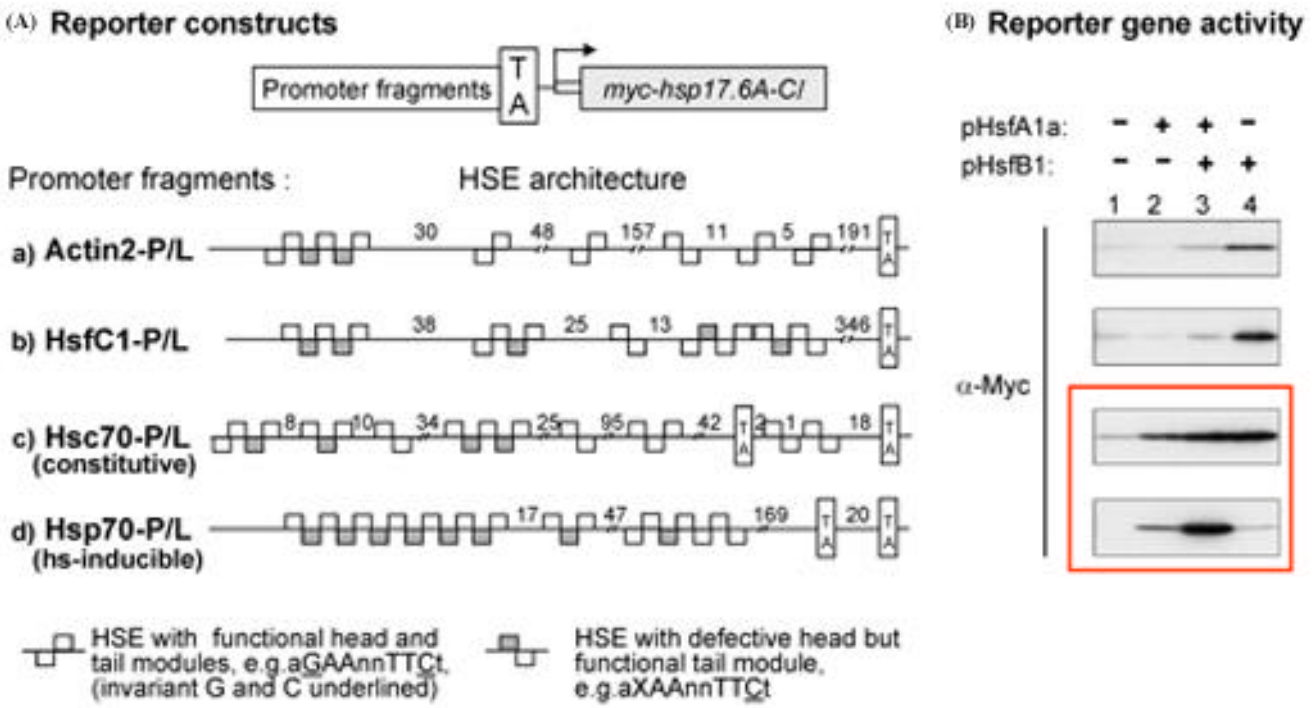

Figure 8. Synergistic gene activation in the presence of HsfA1a and HsfB1 tested for four types of *Arabidopsis* promoters (from Bharti *et al* 2004, modified). (**A**) The reporter constructs contained 1 kb of the promoter/leader sequence with details of the promoter architecture of the actin 2, HsfC1, Hsc70 and Hsp70 genes. For explanations see figure 9 and Nover *et al* (2001). (**B**) Results of reporter assays are based on immunoblots with a Myc-specific antibody. Each reporter construct shows its individual response pattern indicating the decisive role of promoter architecture and the cooperation with other endogenous transcription factors. This is particularly striking when comparing results with the Hsc70- as compared to the Hsp70-derived reporter. For explanations see text.

characterized as repressor of reporter gene expression (Czarnecka-Verner *et al* 2000; Bharti *et al* 2004). A more concise analysis of basic properties of *Arabidopsis* Hsfs can be found in Kotak *et al* (2004).

What do we know at present about biological contexts with Hsfs in other plants?

(i) From sunflower seeds, HsfA9 was isolated and shown to be involved into expression of special chaperone encoding genes during embryogensis and seed ripening. A synergism between HsfA9 and ABA-responsive transcription factors was hypothesized (Rojas *et al* 1999; Almoguera *et al* 2002). Interestingly, in tomato HsfA1a completely disappears during seed ripening and reappears immediately after the onset of germination (S K Mishra and K-D Scharf, unpublished). Bringing the results from both plants together, it is tempting to speculate that HsfA9 replaces HsfA1a in this special developmental stage.

(ii) A mutant in rice with spontaneous necrotic lesions of leaves was identified as a point mutation with Trp > Arg substitution in the DNA binding domain of HsfA4a, which might play a role as antiapoptotic factor (Yamanouchi *et al* 2002). For the corresponding HsfA4 of tomato and *Arabidopsis* we observed that they are strong activators, but they specifically interact with HsfA5 as a type of corepressor (S K Baniwal, unpublished). Hence, the major

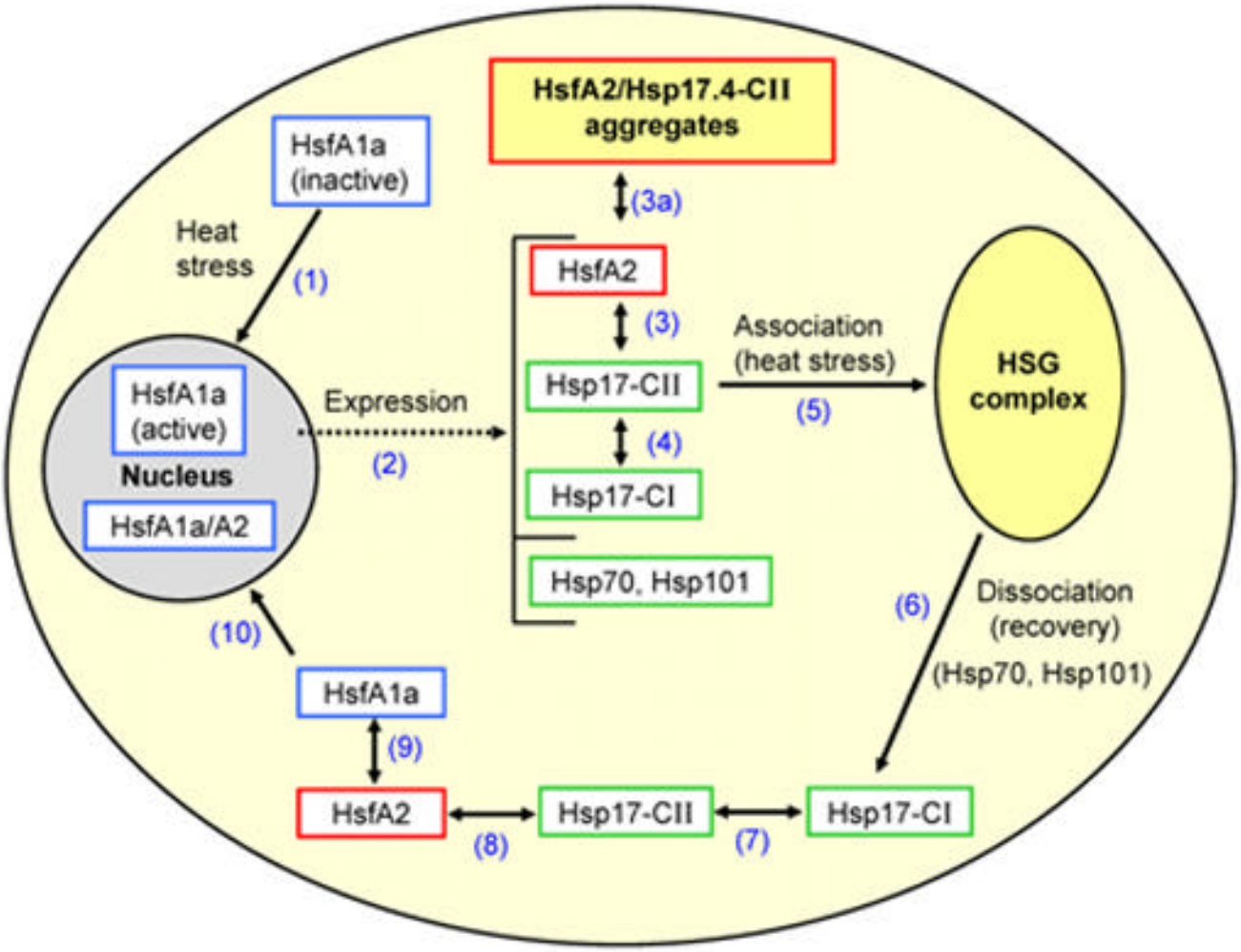

Figure 9. A network of proteins involved in the control of HsfA2 function and intracellular distribution. The master regulator HsfA1a triggers the hs response (step 1) with subsequent expression of Hsps and HsfA2 (step 2). The physical interaction between HsfA2 and Hsp17-CII can result in formation of insoluble aggregates at control temperatures (step 3). This process is counteracted by Hsp17-CI (Port *et al* 2004). Under hs conditions, large cytoplasmic multichaperone complexes (HSG complexes) are formed (step 5) including HsfA2, whose resolubilization in the recovery (step 6) needs the ATP-dependent Hsp70 and Hsp101 chaperone machines. Effective nuclear retention of HsfA2 depends on its soluble state (steps 7, 8) and on the heterooligomerization with HsfA1a (steps 9, 10). For detailed experimental data supporting this model see Port *et al* (2004).

role of the representatives of the HsfA4/A5 group may reside in the control of cell death eventually connected with stress-induced damages.

Evidently, all the data together are only the first pieces of a much more elaborate and biologically intriguing mosaic of Hsfs, chaperones and other co-regulators. Besides the functional diversification of Hsfs, the interaction with other transcription factors on complex promoters (Haralampidis *et al* 2002) was exemplified for HsfB1 with its divergent effects (figure 7). With our increasing knowledge, the Hsfs will become integral parts of the "symphony of transcription factors" for gene expression control as outlined very nicely for a different context by Lemon and Tjian (2000).

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