Heat stress transcription factor HsfA5 as specific repressor of HsfA4

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Unusual abbreviations

aa	amino acid residue
AD	activation domain
AHA	motif containing aromatic, hydrophobic and acidic amino acid residues
BiFC	Bimolecular fluorescence complementation
CaMV	cauliflower mosiac virus
CTAD	C-terminal activation domain
CTD	C-terminal domain
DBD	DNA binding domain
EMSA	Electrophoretic mobility shift assay
EST	expressed sequence tag
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GUS	β-Glucuronidase
HA	Haemagglutinin tag
HTH	helix turn helix
HR-A/B	heptad repeat-A/B
hs	heat stress
HSE	heat stress element
Hsf	Heat stress transcription factor
Hsp	Heat stress protein
Le	*Lycopersicon esculentum*
Lp	*Lycopersicon peruvianum*
LUC	Luciferase
NES	nuclear export signal
NLS	nuclear import signal
Rfu	relative fluorescence units
YFP	Yellow fluorescent protein
Yn	N-terminal part of YFP (aa 1-154)
Yc	C-terminal part of YFP (aa 155-241)

1. INTRODUCTION

1.1. Heat stress response and Hsf proteins

Living organisms respond to environmental stress by triggering orchestrated sets of processes that are critical for normal development and organismic homeostasis. Heat stress response is one such process ubiquitously found in all living organisms. Research on the molecular basis of this response was started by F. Ritossa, he discovered a novel puffing pattern in the polytene chromosomes of the fruit fly *Drosophila buschii* after the application of heat shock (Ritossa, 1962). Central to this response is the new or enhanced synthesis of a set of protective proteins known as heat stress proteins (Hsps). The increase of Hsp synthesis is typically triggered by the binding of heat stress transcription factors (Hsfs) which bind to their target sequences, so called heat stress element (HSE). Hsf bind to the HSE containing promoters of Hsp encoding genes and activate transcription by interacting with components of the transcriptional apparatus (Scharf *et al*. 1998a, Bharti and Nover 2002, Baniwal *et al.* 2004).

Among eukaryotes, heat stress transcription factors display diversity in their number as well as structural characteristics, and their activity patterns differ markedly in response to stress and developmental cues. For instance *Drosophila*, *S. cerevisiae,* and *C. elegans* each has a single Hsf which is essential for survival or normal growth even at normal temperature conditions. However, multiple Hsfs have been reported in vertebrates (*e.g.* Hsf1, Hsf2, Hsf4 in human), and their individual roles during acquisition of thermotolerance and

development have been well documented (Sorger and Pelham 1988, Wiederrecht *et al*. 1988, Sarge *et al.* 1991, Clos *et al*. 1990, Schuetz *et al*. 1991).

1.2 All Hsfs possess highly conserved functional modules

Similar to other transcription factors regulating gene activity, Hsfs posses a modular structure (Fig. 1.1). The key functional modules include DNA binding domain (DBD), oligomerization domain, a flexible linker of variable length connecting them, a nuclear localization signal (NLS) and a C-terminal activation domain (see legends to Fig. 1.1 and Scharf *et al*. 1990, 1998b, Döring *et al*. 2000, Heerklotz *et al*. 2001, reviews Nover *et al*. 2001, Baniwal *et al*. 2004). The

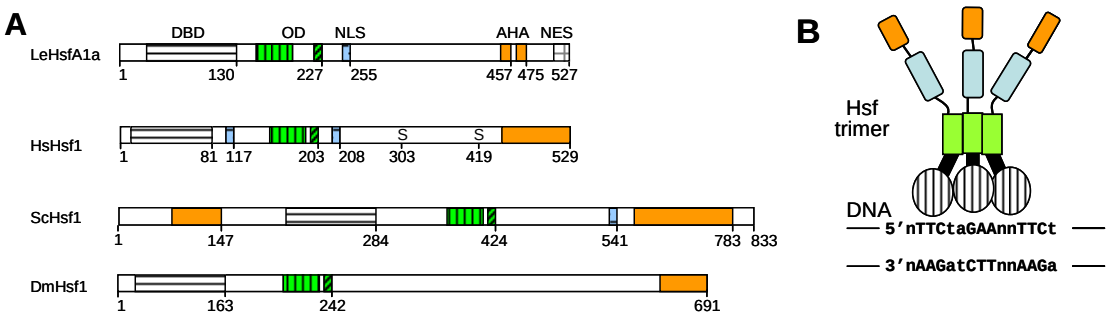


Figure 1.1 Basic structure of Hsf proteins from different eukaryotic organisms and Hsf binding to HSE.

A) (1) The central part of the DBD is the helix-turn-helix motif (H2-T-H3) with the considerable number of amino acid residues invariant among different organisms. (2) The oligomerization domain (OD) containing heptad pattern of hydrophobic residues called HR-A and HR-B, in plants they are separated from each other by a linker (21 amino acid residues) (3) The NLS represents a cluster of basic residues (K, R) recognized by the NLS receptor. (4) Central elements of the activator region (marked orange) are one or two short motifs (AHA motifs) rich in aromatic (W, Y, F), hydrophobic (L, I, V), and acidic (D, E) amino acid residues. (5) A Leucine-rich motif at the C-terminus functions as an NES. Abbreviations used: Le, *Lycopersicum esculentum*; Hs, *Homo sapiens;* Sc, *Saccharomyces cerevisiae*; Dm, *Drosophila melanogaster*.

B) Hsf binding to HSE (palindrome GAAnnTTC) as trimeric protein through their DNA binding domains.

AHA motifs of plant Hsfs are the contact sites to interact with the components of the transcriptional machinery (Döring *et al*. 2000). Similar motifs, have also been defined in other transcription activators, *e.g.* yeast Hsf, Gal4, and Gcn4, or vertebrate Hsf1, p53, VP16, Fos, Jun RelA, Sp1, C/EBPa, and E2A (for a summary see Nover and Scharf 1997).

1.3 Regulation of Hsf transcriptional activity

Before discussing details of the plant Hsf system, it is useful to briefly summarize results about human Hsf1 because it has been extensively characterized. Some fundamental aspects of HsHsf1 function as gene activator are also applicable to plant Hsfs.

1.3.1 Post translational modifications

Under non-stress conditions, majority of Hsf1 localizes in the cytoplasm as an inactive monomer by chaperone proteins and intramolecular interactions. Multistep activation pathways lead to the formation of DNA binding Hsf1 trimers which further acquire transcriptional competence by modifications (Cotto *et al.* 1997, Voellmy 2004). Detailed analyses of these post translation modification events revealed phosphorylation and sumoylation at multiple sites. Phosphorylation of Ser residues at positions 303, 307, 308 contribute to the inactive state of Hsf1, whereas phosphorylation at positions 230, 326 and 419 favour its active state (Holmberg *et al*. 2001, Kim *et al*. 2005, Guettouche *et al*. 2005). In addition, the Lys residue at position 298 is sumoylated only if Ser residues at positions 303 and 307 are phosphorylated. However, the mechanisms by which these modifications control the transcriptional activity of Hsf1 remain elusive (Sarge *et al*. 1993, Cotto *et al*. 1996, Hong *et al*. 2001,

Hietakangas *et al*. 2003). In addition, oxidation of Cys in the DBD regulates activity in response to heat stress and oxidative stress conditions (Ahn and Thiele 2003). Residues 201-330 of Hsf1 have been characterized as “regulatory domain” negatively regulating the Hsf1 activity (Voellmy 2004).

1.3.2 Ribonucleoprotein complex

Recently, a ribonucleoprotein complex formed of translation elongation factor eEF1A and non-coding RNA, HSR1 (heat shock RNA-1) were identified to control the hs-response by regulating Hsf1 activity (Shamovsky *et al*. 2006). Association of Hsf1 into this complex was dramatically enhanced by hs. Knock-down of HSR1 in transient reporter assays strongly inhibited Hsf1 DNA binding and transcriptional activity. Both, eEF1A and HSR1 are constitutively expressed. The following mechanism was postulated: Because of translation shut down in response to heat stress (Panniers 1994) more of eEF1A (as HSR1-eEF1A complex) becomes available. It captures Hsf1 released from the inhibitory Hsp90 containing complexes and assists its assembly into trimers and/or increase the stability of transcriptionally competent Hsf1 trimers.

1.3.3. Molecular chaperones

In its inactive state Hsf1 exists in a multichaperone complex similar to that reported for steroid receptors (Pratt and Toft 1997). In the hs-recovery phase Hsf1 activity is down regulated *via* a feed-back mechanism by binding to chaperones Hsp90 and Hsp70. Hsp70 and a co-chaperone Hdj1 were found to directly bind to the activation domain of Hsf1. The repressor function was proposed to be due to the inaccessibility of the Hsf1 activation domain to the transcriptional machinery (Shi *et al*. 1998). In contrast to this, the Hsp90

containing complex (Hsp90-p23-immunophilin) retained Hsf1 in the inactive, cytoplasmically localized, nontrimeric state which poorly binds to DNA (Zou *et al*. 1998). It is hypothesized that initial formation of the Hsf1-Hsp/c70 complex in the nucleus leads to the restoration of the cytoplasmic Hsp90 containing complex (Shi *et al*. 1998).

1.4 Heat shock factor binding protein (HSBP)

A new level of Hsf regulation was discovered in 1998 by small Hsf binding proteins (HSBP). These proteins are ~80 aa residues long and they interfere with the heat shock induced trimer formation of Hsf1 (Satyal *et al*. 1998). HSBP are solely composed of coiled-coils (Li-Jung *et al*. 2002). A protein with similar function was described for maize. The gene *empty pericarp2* (*emp2*) encodes a paralog of HSBP1 (Fu *et al.* 2002). A mutation that abolished emp2 expression resulted in retarded embryo development and early-stage abortion of embryogenesis most likely because of uncontrolled hs response (Fig. 1.2). Evidently, uncontrolled overproduction of Hsps interferes with normal development.

Based on yeast two hybrid interaction tests, the two maize HSBPs were shown to interact selectively with few maize class A Hsfs, *i.e.* mainly Hsfs A2c and A4a for HSBP2 and Hsfs A2e, A3, A4d and A5 for EMP2. Moreover, mutations in few conserved hydrophobic residues in the HsfA4a HR-A/B region abolished the interaction with HSBP2 (Fu *et al*. 2005).

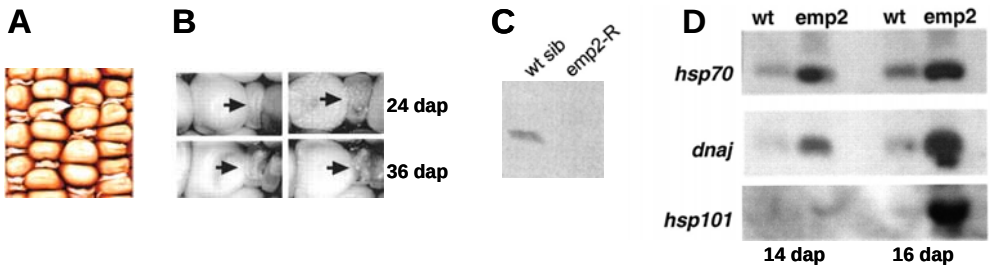


Figure 1.2 empty pericarp2 encodes a negative regulator of the hs response and is required for maize embryogenesis.

A) Self-pollinated ears of emp2-R/Emp2 heterozygous plants segregate 1:4 collapsed, defective-kernel phenotypes.

(B) Endosperm development in emp2 mutant kernels. Although kernel filling does occur at earlier stage (24 DAP) in emp2-R mutant seeds, endosperm development eventually is aborted. At 36 DAP, much of the endosperm material that has accumulated in mutant kernels has been reabsorbed, and the mutant kernels collapse.

C) EMP2 protein does not accumulate in null emp2 mutant kernels. Protein blot analyses with EMP2-specific antibody from non-mutant sibling (wt sib) emp2-R mutant kernels at 12 DAP.

D) RNA Gel Blot analyses of emp2 mutant kernels. At 14 and 16 DAP, transcripts homologous with the heat shock genes hsp70, dnaj, and emp2 are overly abundant in emp2 mutant kernels, whereas hsp101 transcripts accumulate at 16 DAP. (Figure modified from Fu *et al.* 2002)

1.5 Characteristic features of heat stress transcription factor proteins in plants

In contrast to animals, plants possess extraordinarily large Hsf families with >20 members (Nover *et al*. 2001, Baniwal *et al*. 2004). It may be argued that plants being sessile, need a much more robust and diversified system to maintain their physiological homeostasis during multiple stress situations in their surroundings *e.g.* heat stress combined with oxidative stress, water deficiency, and nutrient deprivation. Plant Hsfs have been classified according to their oligomerization domain which is built of two parts, HR-A and HR-B. Similar to all non-plant Hsfs both parts may be immediately adjacent (class B), or they are

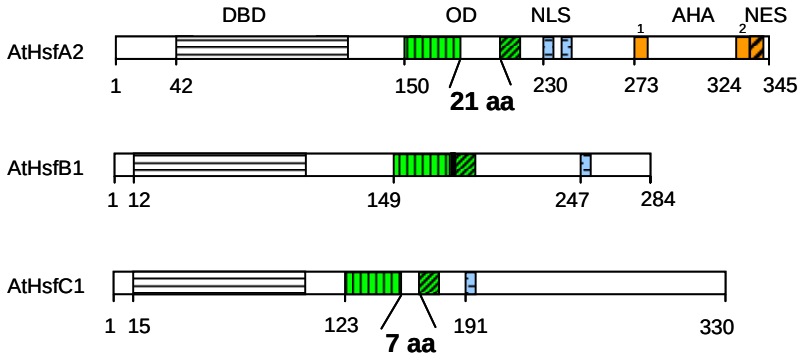


Figure 1.3 Classification of plant Hsf proteins into three classes.
Block diagrams representing three Hsfs from each class of Arabidopsis Hsf family. Classes A, B, and C are represented by HsfA2, HsfB1, and HsfC1 respectively. (For more details refer to Fig. 1.1)

separated by insertion of 21 (class A) and 7 amino acid residues (class C) (Fig. 1.3) (Nover *et al*. 2001). Hsfs from Class A and Class B have different transcriptional activation properties and the members of the two classes do not physically interact directly. Recently, Chan *et al.* 2007 provided evidence that the linker region together with HR-B defines the specificity of interaction between Hsfs.

1.6 Multiplicity of plant Hsfs: Redundancy *versus* diversification of function

Although our understanding of the complexity of the plant Hsf family is very limited, functional diversification seems to be the main reason for the coexistence of more than 20 Hsfs in plants. Investigations on the structural and functional diversification so far are restricted to Hsfs A1a, A2, A3, A4d, A9 and B1. The results are briefly described below.

1.6.1 HsfA1a as master regulator of thermotolerance in tomato

HsfA1a is the master regulator of heat stress response in tomato plants and it can not be replaced by any of the other Hsfs (Mishra *et al*. 2002). Analysis of different tissues from HsfA1a co-suppression lines (CS2 and CS3) revealed marked reduction in the expression of Hsfs A2 and B1. Furthermore, the transcript and protein levels of Hsp104 and small heat stress proteins correlated directly with HsfA1a expression state (Fig. 1.4*A* and *B*). As a result of the down-regulation of hs-inducible Hsfs and Hsps, these plants were highly vulnerable to elevated temperatures. Following a treatment for 1 h at 45°C, CS plants in contrast to wt and OE plants, never recovered and finally succumbed to death (Fig. 1.4*C*). It is important to mention that at normal growth temperature, CS plants behave identical to wild type and HsfA1a over-expression (OE) plants. This underlines the unique role of HsfA1a for the acquisition of heat stress tolerance in tomato plants.

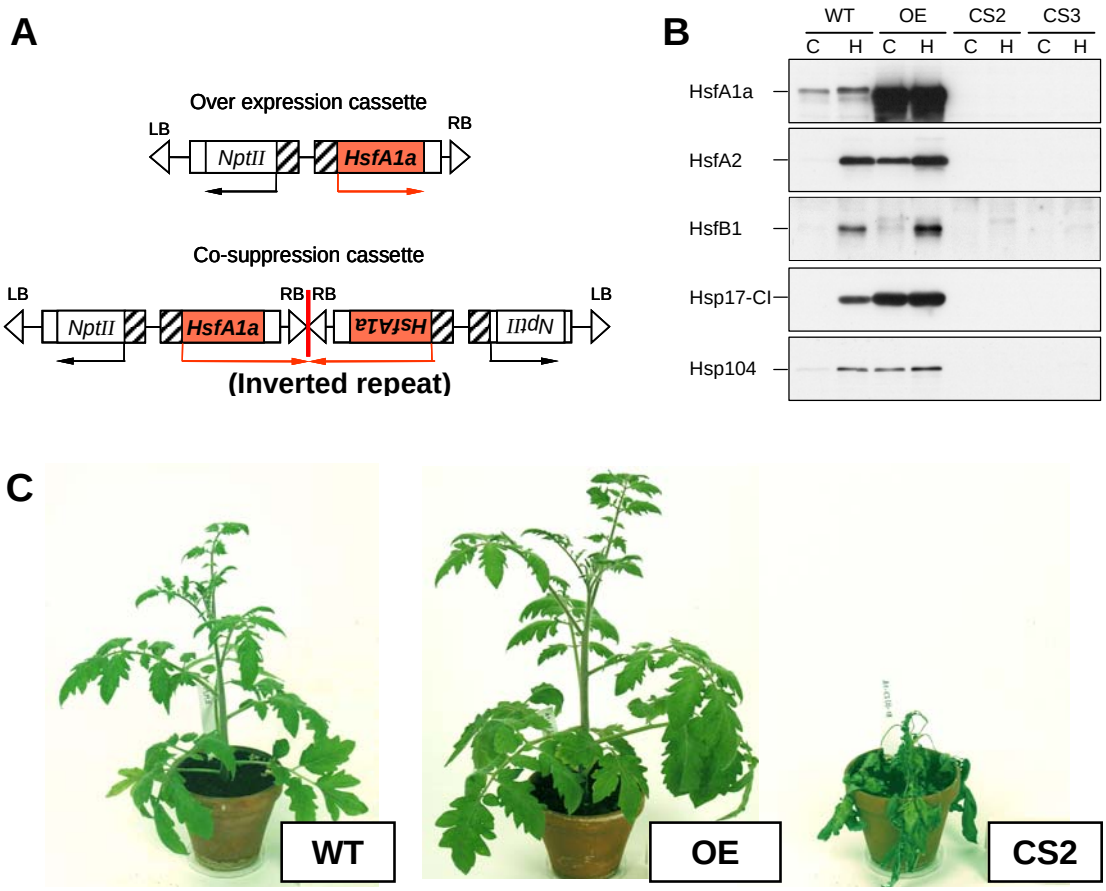


Figure 1.4 LeHsfA1a is the master regulator of heat stress response in tomato plants.

A) T-DNA based expression cassettes for constitutive HsfA1a overexpression (OE) and co-suppression (CS). Expression of HsfA1a from both cassettes was driven by cauliflower mosaic virus 35S promotor (CaMV).

B) Protein blots analysis for various components of heat stress response system using specific antisera for them (see materials and methods).

C) Five week old plants from wild type (WT), HsfA1a over-expression and co-suppression tomato lines were heat stressed for 1 h at 45°C and pictures were taken after 1 week of recovery at room temperature. (Figure modified from Mishra *et al*. 2002).

Surprisingly, in Arabidopsis knockout of HsfA1a and HsfA1b neither alone nor together markedly affected the long-term thermotolerance (Lohmann *et al.* 2004). In this context it may be risky to state “HsfA1a is the master regulator of heat stress response” in general. It will be interesting to analyze whether in

Arabidopsis, other A1-type Hsfs serve as master regulator or whether this function is rather fulfilled by multiple A1 type Hsfs.

1.6.2 HsfB1 as co-regulator of heat stress as well as house-keeping genes

So far, tomato HsfB1 is the only member of plant class B Hsfs that has been analyzed in detail. HsfB1 has no activator function of its own but together with acidic activators (*e.g.* HsfA1a), it synergistically enhances the expression of target genes (Bharti *et al*. 2004). One of the key features of this phenomenon was the ability of HsfB1 to recruit histone acetyl transferase CBP by virtue of its histone like motif (GRGK motif), into an enhanceosome like structure (Fig. 1.5). Mutation of the Lys residue in this motif disrupted the co-activator function of HsfB1. Although HsfB1 did not directly interact with HsfA1a, they form high molecular weight complexes due to the scaffolding function of CBP, which interacted with HsfB1 *via* GRGK motif and HsfA1a *via* AHA motifs. At target gene promoters intermingled clusters of perfect and imperfect HSE were found to be a prerequisite for the joint binding of HsfB1 with HsfA1a/Acidic Activators.

1.6.3 HsfA2 is exclusively expressed under hs-conditions and has a dominant role

HsfA2 is the most extensively characterized Hsf of plants. In both, Arabidopsis and tomato HsfA2 accumulates to fairly high levels following multiple cycles of heat stress and recovery (Port *et al.* 2004). Following are the essential points obtained from several studies to characterize HsfA2 (Fig. 1.6).

- Due to functionally dominant NES over NLS tomato HsfA2 shuttles between nucleus and cytoplasm (Heerklotz *et al.* 2001). However, hetero-oligomerization of HsfA2 with HsfA1a leads to its nuclear retention (Scharf *et*

al. 2000). Moreover, both together function synergistically as a type of super-activators for transcription of Hsp genes (Chan *et al*. 2007).

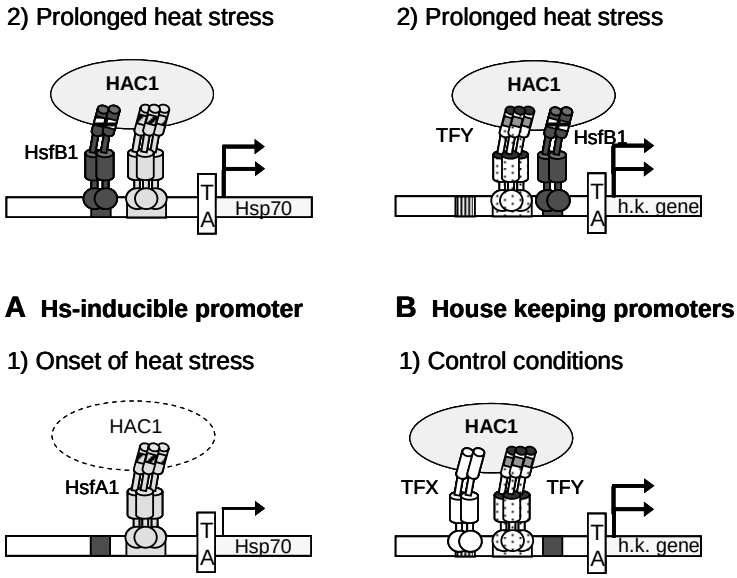


Figure 1.5 Model depicting the interplay of HsfB1 with other activators.
Different stages of heat stress involve a complex interplay of HsfB1 with acidic activators (HsfA1a, TFX, TFY) and co-recruitment of HAC1. (Figure modified from Bharti *et al*. 2004).

- In Arabidopsis and tomato, Hsp17.4-CII binds to the C-terminal activator domain of HsfA2 and this affects its aggregation state, intracellular localization and activator function (Port *et al*. 2004). Since this interaction could be modulated by Hsp17-CI and/or HsfA1a it may be speculated that all these proteins form a regulatory network which control HsfA2 activity (Fig. 1.6).

- Studies with knock-out and overexpression transgenic lines for HsfA2 in Arabidopsis could identify many of its target genes (Charng *et al*. 2006, Nishizawa *et al*. 2006, Schramm *et al*. 2005, Chunguang *et al*. 2005). Among

them were various Hsp and non-Hsp (*e.g. Apx2*) genes suggesting the crucial involvement of HsfA2 in maintaining the cellular homeostasis in Arabidopsis under conditions of environmental stress.

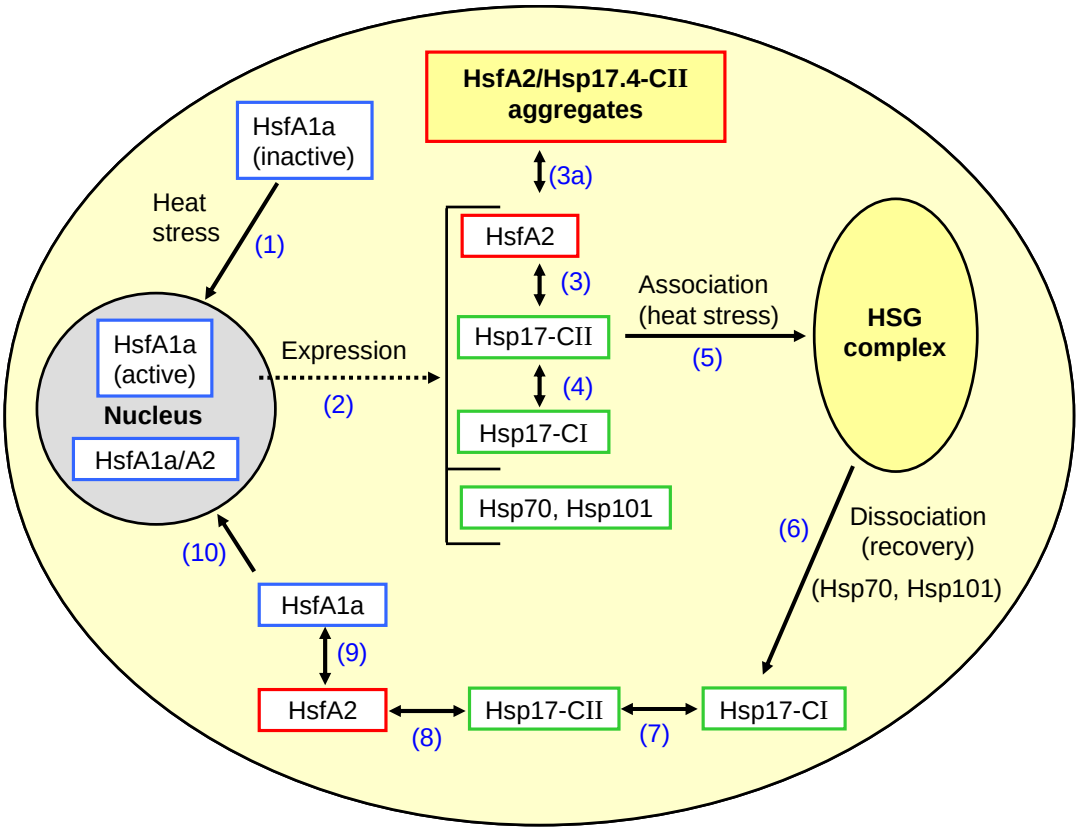


Figure 1.6 A network of proteins involved in the control of HsfA2 function and intracellular distribution.

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.

1.6.4 HsfA9 is exclusively expressed during late stages of seed development

Jordano and colleagues used yeast mono hybrid system with the promoter fragment of a developmentally regulated soybean *hsp17.6C1* gene to isolate a new Hsf (*HsfA9*) whose expression was exclusively detectable during late stages of seed development (Almoguera *et al*. 2002). These findings were considerably enlarged by Kotak *et al.* (2007) showing that *ABI3* (abscisic acid insensitive locus 3) transcription factor acts as regulator of *HsfA9* expression which in turn regulates the expression of a specific subset of Hsp genes during seed maturation in Arabidopsis (Fig. 1.7).

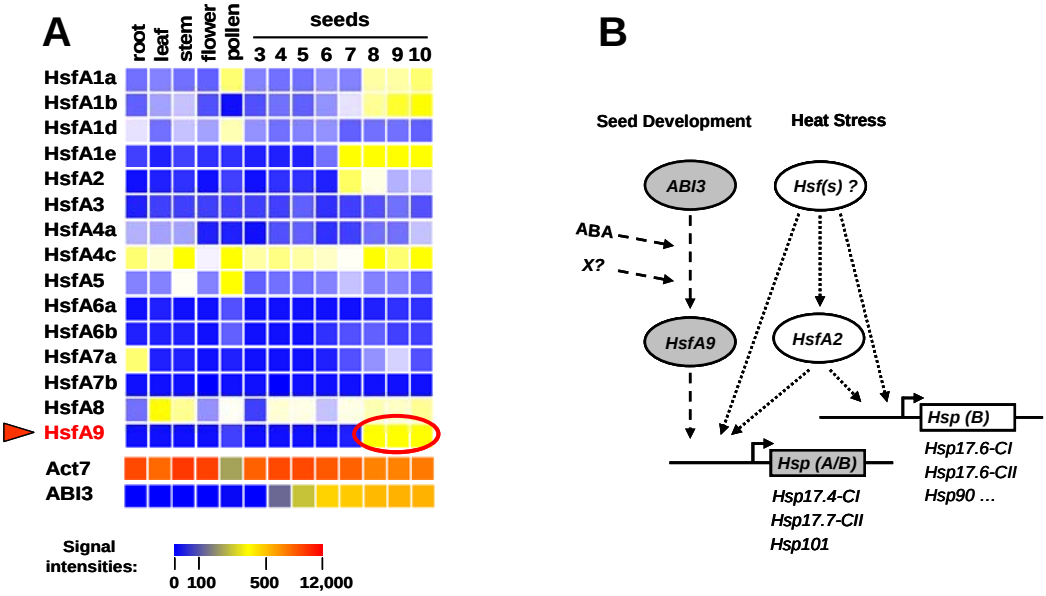


Figure 1.7 Microarray analysis and model representing the cascade involving ABI3, HsfA9, and Hsps.

A) Expression profiles of Hsfs in different tissues and developmental stages of Arabidopsis representing expression pattern of Hsfs and regulators involved in seed development.

B) Model for the regulation of *Hsp* genes in seeds and the role of Hsfs. HsfA9 expression regulated by ABI3 which requires abscisic acid (ABA) or a yet unknown discrete developmental signal. HsfA9 in turn acts as the transcriptional regulator of the developmentally expressed subset of Hsps. Independent of HsfA9, the same subset of *Hsps* is transcriptionally induced by heat

stress as are many other *Hsp* genes that are not developmentally regulated. (Figure modified from Kotak *et al.* 2007).

1.6.5 HsfA4 as anti-apoptotic factor and regulator of redox-regulated gene expression

An interesting observation about HsfA4 was made in rice when a mutant in *Spl7* gene (spotted leaf) was found and identified as a point mutation in the DBD of HsfA4d (Yamanouchi *et al.* 2002). These *Spl7* mutant plants develop hypersensitive response and cell death when they are challenged with very mild stress conditions (Fig. 1.8). Examples for such daily stress effects on leaves are certainly connected with high light intensities and the generation of reactive oxygen species. Interestingly seedlings and young leaves were not affected with any of the mentioned stress conditions. Furthermore, there was a direct correlation between the susceptibility for lesion development and the age of the *Spl7* mutant plant.

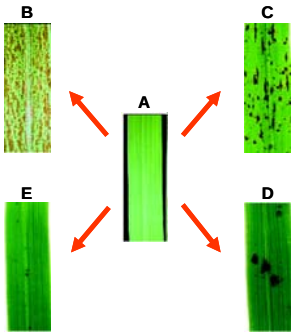


Figure 1.8 Lesion-mimic phenotype of the *spl7* mutant (HsfA4d) of rice.
Pictures of leaves from 2-month-old plants grown under various conditions. A) Wild type, B-E *Spl7* mutant after following treatments, B) Natural summer field (30–35^{o}C), C) Green-house (26^{o}C, solar radiation), D) Growth chamber (35^{o}C, artificial light), E) Green-house (26^{o}C, UV-filtered solar radiation). (Figure modified from Yamanouchi *et al*. 2002).

In another report, function of HsfA4 was postulated to regulate gene expression in response to redox stress (Davletova *et al*. 2005). In transgenic

Arabidopsis plants over-expressing a dominant negative form of HsfA4a the accumulation of transcripts encoding APX1 and Zat12 was prevented. The molecular mechanisms underlying these specialized functions of HsfA4d are not yet clear.

1.7 Objectives of the thesis

Objectives of the thesis are focused on the functional characterization of two Hsfs, HsfA4 and HsfA5 from tomato and Arabidopsis. The two Hsfs represent a pair where HsfA4 is a activator and HsfA5 is a specific repressor. The results will be represented in the following parts.

- Elaboration of tomato Hsf network and of the evolutionary relations within the A4/A5 Hsf subgroup. Definition of the signature sequences.
- Molecular characterization of HsfA4 and HsfA5 from tomato and Arabidopsis in terms of their expression and function as transcriptional activators.
- Investigate the activator/repressor relationship between the two Hsfs.
- Functional anatomy of HsfA5 as specific repressor of HsfA4 activity.
- Intracellular localization of the individual proteins as well as of their hetero-oligomeric complexes.
- Characterization of the oligomerization domain for selective and preferential formation of hetero-oligomers between HsfA4 and HsfA5.

2. MATERIALS AND METHODS

2.1 General reagents and procedures

Standard protocols were used for cloning and nucleic acid analysis (Ausubel *et al*. 1993, Sambrook *et al*. 2001). Total RNA was prepared from plant tissues by using the RNeasy® plant mini kit (Qiagen, Hilden, Germany). For cDNA synthesis Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas, St. Leon-Rot, Germany) was used according to the manufacturer's protocol. PCR fragments for subcloning were generated by using the High Fidelity PCR Enzyme Mix (Fermentas).

Protein extraction, SDS-PAGE, and protein blotting analysis were performed as described (Mishra *et al*. 2002, Port *et al*. 2004). The generation and use of specific antisera against individual tomato (*Lycopersicon esculentum*) Hsfs (HsfA1a, A2, A3) and Hsp17-CI were described before (Lyck *et al*. 1997, Bharti *et al*. 2000, Port *et al*. 2004). Primary antibodies for immunodetection of green fluorescent protein (GFP), Myc, HA, and Strep -tagged proteins were obtained from Roche Diagnostics (Mannheim, Germany), HISS Diagnostics (Freiburg, Germany), and IBA (Göttingen, Germany). Horse radish peroxidase (HRP) -conjugated secondary antibodies were obtained from Sigma-Aldrich (Taufkirchen, Germany).

For transient gene expression studies, tobacco (*Nicotiana plumbaginifolia*) leaf mesophyll protoplasts were used. Polyethylene glycol (PEG)–mediated co-transformation of reporter and Hsf expression plasmids was carried out as described previously (Döring *et al*. 2000, Scharf *et al*. 1998, Treuter *et al*.

1993). *E. coli* BL21-CodonPlus® (DE3)-RIL cells (Stratagene, Amsterdam) were used for overexpression of recombinant Hsfs and GST fusion proteins.

2.2 Plasmid constructs for transient expression studies in protoplasts.

The Hsf-dependent reporter plasmids p*Gmhsp17.3B-CI::GUS* and p*HSE9::GUS* and the repressor reporter construct p35S::HSE9-*GUS* were described before (Treuter at al. 1993). Plasmid constructs for Hsf expression in plant cells are based on the pRT series of vectors (Töpfer *et al*. 1988). Constructs for Hsfs A1, A2, and A3 were described before (Scharf *et al*. 1998, Bharti *et al*. 2000). PCR fragments containing the full length ORF regions of Hsfs tested in this study were generated by using cDNA preparations from sepals of opening flower buds (HsfA4b) or young leaves (HsfA5) as template. Gene-specific oligonucleotide primers were designed on the basis of corresponding EST sequence data (see Table S*2*) and adapted for introducing appropriate restriction sites for in-frame subcloning of amplified DNA fragments into pRT vectors providing the corresponding sequences for affinity tags (Kirschner *et al*. 2000, Siddique *et al*. 2003). Further deletions or modifications were done on the basis of these parental expression vectors. An overview of all constructs and primer sequences are compiled in Table S*2*). For subcellular localization studies, PCR fragments of the corresponding Hsfs were subcloned into p35dS::*GFP* to generate in-frame GFP-Hsf fusions. The binary BiFC plant transformation vectors *pSPYNE* and *pSPYCE* (Walter *et al*. 2004) were kindly provided by Klaus Harter (CPMB, University of Tübingen, Germany) and were used as template DNA for PCR amplification of Myc-YN and HA-YC encoding sequences for cloning into pRT vectors in order to

achieve compatible cloning sites to create Hsf-Myc-YN and Hsf-HA-YC fusion constructs (see Supplemental Table S*2*).

2.3 Expression constructs for yeast and *E. coli.*

Growth and transformation of yeast strains and selection on 5-Fluoroorotic Acid (FOA) followed standard protocols (Ausubel *et al*. 1993, Rose *et al*. 1990). For expression of tomato Hsf proteins in yeast cells, the corresponding DNA fragment were subcloned from plant expression constructs into pAD5Δ (Boscheinen *et al*. 1997). *Saccharomyces cerevisiae* (haploid strain, RSY4) used for functional Hsf substitutions was derived from strain RSY10 (MATa/MATa, ade2/ade2, ade6, can1/can1, his3,11,15/his3,11,15, leu2-3, 112/leu2-3,112, trp1-1/trp1-1, ura3-1/ura3-1), a kind gift of Dr. Mark Vidal (Northwestern University, Evanston, IL.).

Constructs for expression in *E.coli* for GST-pull-down bait proteins were based on plasmid pGEX-4T-1 (Amersham Biosciences, Freiburg, Germany) and generated by in-frame fusions of DNA fragments encoding C-terminal parts of tomato Hsfs A4b (aa residues 112-393) and A5 (aa residues 110-478), respectively. Full length HsfA1a, HsfA1a(OD:A4b) and 3HA-HsfA5 encoding sequences were cloned in pJC vectors (Bharti *et al*. 2004).

2.4 GST pull-down interaction assay

Bait proteins were purified with the GST Purification Module (Amersham Biosciences) according to the manufacturer's protocol. The pull-down assay was conducted in 20 mM Tris pH 7.5, 150 mM NaCl, 5mM $MgCl_2$, 0.01% NP40 (supplemented with Complete™ Protease Inhibitor, Roche Diagnostics)

by incubation of GST-Hsfs bound to Glutathion-Sepharose with lysates prepared either from yeast cells expressing HsfA4b-Strep or from *E. coli* cells expressing HsfA1a or 3HA-HsfA5, respectively. The bound proteins were eluted and separated by SDS-PAGE and detected with the appropriate antibodies by protein blot analysis.

2.5 Yeast two-hybrid interaction assay

For two hybrid interaction studies the pGal4-BD bait and pGal4-AD prey vector system (Stratagene) was used as described previously (Scharf *et al*. 1998, Bharti *et al*. 2000). All *Arabidopsis* Hsf fusion constructs used in this study were described earlier (Kotak *et al*. 2004) and were kindly provided by Pascal von Koskull-Döring (Goethe-University, Frankfurt). The strength of interaction was confirmed by colony growth in presence of the histidine biosynthesis inhibitor 3-aminotriazole (3AT).

2.6 Localization and interaction studies in tobacco protoplasts

Tobacco protoplasts transformed with appropriate combinations of plasmids encoding chimeras of Hsfs with GFP or with YFP domains were analysed after 16 h of expression. For nuclear retention of Hsf proteins, protoplasts were incubated in presence of 20 ng ml^{-1} leptomycin B (LMB, kindly provided by Minoru Yoshida, Tokyo) added 3 h before harvesting (Heerklotz *et al*. 2001). Fluorescence microscopy analysis of protoplasts transformed with GFP-Hsf fusion constructs was performed immediately after harvesting without any further treatments. Fluorescence light emission of recombined YFP in hetero-oligomeric Hsf-YN/Hsf-YC complexes was determined after fixation of

protoplasts and staining with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) to visualize the nuclei (Heerklotz *et al*. 2001).

For fluorescence microscopic analysis a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) was used combined with a Color View XS photo system (Soft Imaging System, Münster, Germany). For overlay, captured images were resized and combined by using Photoshop 8.0 Software (Adobe Systems, La Jolla, CA).

3. RESULTS

3.1 Identification of new tomato heat stress transcription factors

The technological advancements in the area of molecular biology and information technology have made it feasible to rapidly clone sequence and analyze DNA molecules. This is the basis for the generation of large EST (expressed sequence tag) libraries. Such libraries are generated by randomly sequencing a large population of cDNA clones derived from the whole set of mRNA from a given tissue. EST libraries are available for a number of important cultural plants including tomato, Arabidopsis, rice, potato, medicago and others (www.tigr.org/tdb/tgi/lgi/index .html). Despite the general drawback for our aim that these libraries were not derived from heat stressed plant or tissues, searches in the rapidly growing databases proved to be very useful.

The presence of highly conserved sequence motifs, so called signature motifs, in Hsfs allowed us to identify a great number of ESTs encoding >18 tomato Hsfs, including ESTs for the four Hsfs cloned and sequenced previously from the closely related *L. peruvianum*, *i.e.* HsfA1a (former HsfA1), HsfA2, HsfA3, and HsfB1 (Fig. 3.1) (Scharf *et al*. 1990; Bharti *et al*. 2000). The analyses indicated that the ESTs representing Hsfs A1a, A5, A6b, B1 and C1 are detected much more frequently than others (S. Baniwal, Diploma thesis 2002), and in contrast Hsfs A4b and B3 are represented by only one or two ESTs. If the frequency of EST detection in the database is considered as direct indicator for its expression level then Hsfs A1a, A6b, B1, and C1 are most frequently expressed. It should be noted however, that

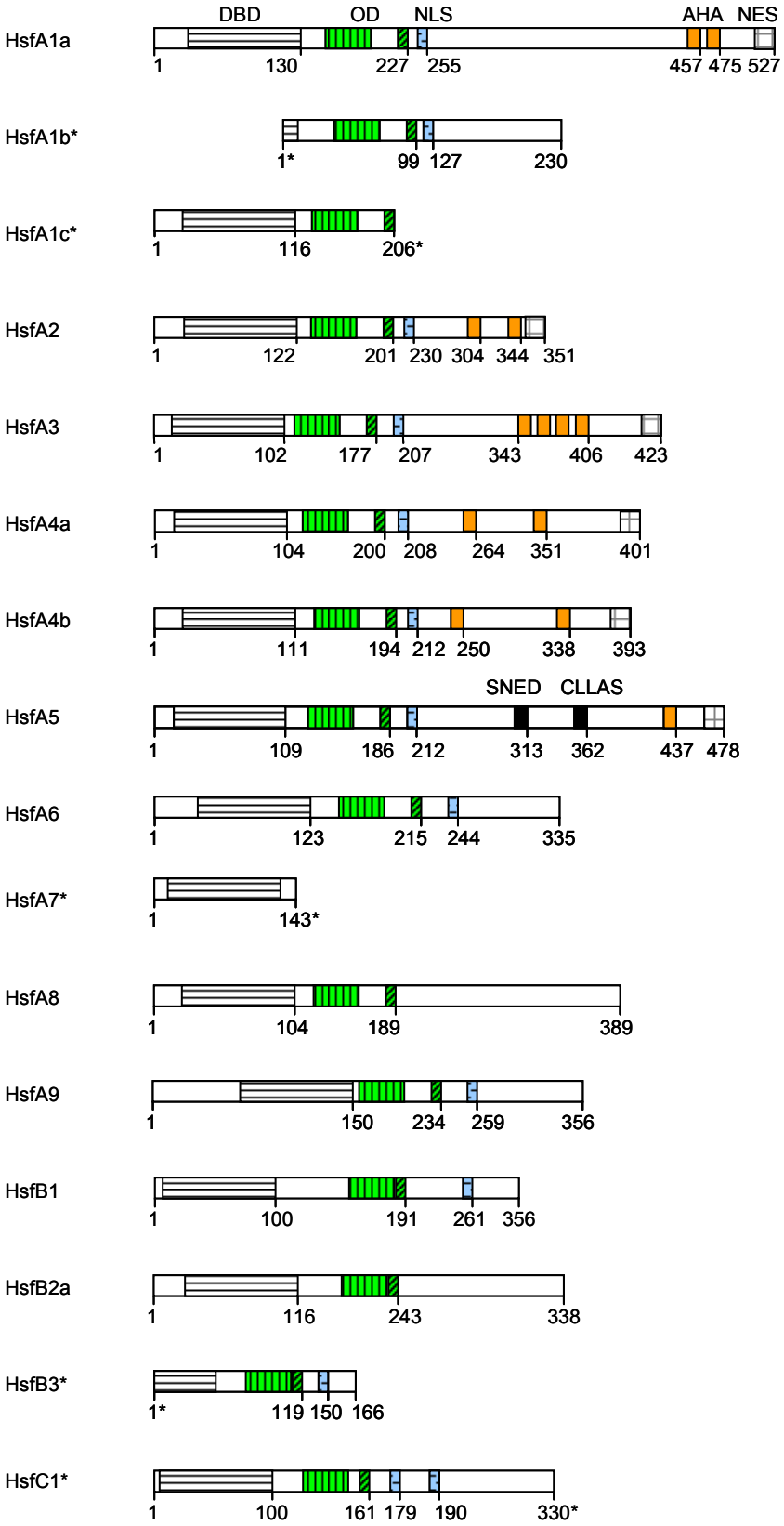


Figure 3.1 Block diagrams for all tomato Hsfs identified so far.

Details of each functional domain are mentioned in Fig. 1.1. (*) indicates that the EST clone was partial and represent only the indicated parts of Hsf. The numbers mark the position of the amino acid residues.

some Hsfs may not be represented at all in the present EST database because their transcripts are only found in specific tissues or under certain conditions. Examples are HsfA2 with massive expression only after hs induction or HsfA9 with exclusive expression in developing seeds.

Bioinformatics tools such as Clustal 1-8_msw, permit easy and extensive sequence comparison. Unfortunately, some of the tomato Hsf ESTs represent only a part of the full length mRNA and this certainly affects the quality of sequence comparison. To minimize this problem, the Arabidopsis Hsf family with 21 representatives and rice (*Oryza sativa)* Hsf family with 23 representatives were simultaneously utilized to assign the correct positions of tomato Hsfs in the phylogenetic tree (Fig. 3.2) (Nover *et al*. 2001; Baniwal *et al*. 2004).

3.2 Phylogenetic analysis of the HsfA4/A5 subgroup

Usually the highly conserved N-terminal parts of the Hsf with the DBD and OD were used to identify Hsf encoding ESTs. Unfortunately, this excludes many unidentified ESTs encoding C-terminal parts of Hsfs. With the increasing number of Hsfs from different plants, we developed signature sequences also for the C-terminal activation domain of Hsfs (Kotak *et al*. 2001). Using these more elaborate tools, we compiled data about two special subgroups of Hsfs which will be at the centre of this thesis. These are Hsfs A4 and HsfA5.

HsfA4	NLS			AHA-region			NES
LeA4b	ASRKRRLL	113 aa	NDVFWQQFL	TETPGCTEPQQVENKGINE	12 aa	YWWNRGVNLENLAERMGHLSSPATGS*	
AtA4c	HERRKRRF	83 aa	NDDFWEQCL	TENPGSTE-QQEVQSERRD	13 aa	YWWNSGNVNNIT---------EKAS*	
AtA4a	NERKRRFP	127 aa	NDGFWQQFF	SENPGSTE-QREVQLERKD	11 aa	CWWNSRNVNAITEQ-LGHLTSSERS*	
LeA4a	NERKRRLP	130 aa	NDVFWEQFL	TENPGSTDVKPEREDMESK	11 aa	FWWNRKTVISLTEQ-LGHLTPAE*	
OsA4a	HRKKRRLP	167 aa	NDGFWQQFL	TEQPGSSDAHQEAQSERRD	15 aa	LWWGKRNVEQITEK-LGLLTSTEKT*	
OsA4d	FSKKRRVP	180 aa	NDVFWERFL	TETP	11 aa	SPKDDVKAELGCNGFHHREKVDQITEQMGHLASAEQTLHT*	

HsfA5	NLS		SNED		CLLLAS		AHA-region		(NES)
LeA5	FSKKRRLP	52 aa	STQSSNED	78 aa	CQLNLSLAS	63 aa	NDVFWEQFL	TERPGCSDNEEASS	23 aa RKVEHLTL*
AtA5	YNKKRRLP	53 aa	SIQSSNEE	68 aa	CHLNLTLAS	55 aa	NDVFWEQFL	TERPGSSDNEEASS	23 aa KNIEQLTL*
OsA5	FNKKRRLP	54 aa	STQSSNED	54 aa	CHLSLTLAS	75 aa	NDKFWEQFL	TERPGCSETEEASS	24 aa EDVEQLKL*

Table 3.1 C-terminal signature sequences of Hsfs A4 and A5

Amino acid sequence alignment of the C-terminal domains of HsfA4 and HsfA5 from Arabidopsis, rice, and tomato, showing the C-terminal signature sequences. The numbers in between the amino acid sequence refer to the number of non-conserved residues. SNED and CLLEAS are the boxes that contain invariable amino acid residues among all plant HsfA5. The predicted AHA motif is marked with red box. * indicates stop codon. (NES) resembles a typical NES but does not function as NES as found by experiment analyses.

The analysis revealed presence of a single HsfA5 and usually two A4-type Hsfs (Fig. 3.3 and Table 3.2). Owing to characteristics of their N-terminal and C-terminal signature sequences, Hsfs A4 and A5 are clearly separate from the other

members of the Hsf family (Fig. 3.2). This indicates that Hsfs A4 and A5 are phylogenetically most closely related to each other than to the other members. Moreover among different plants, HsfA5 subgroup members are much more conserved among each other than the members of the HsfA4 subgroup.

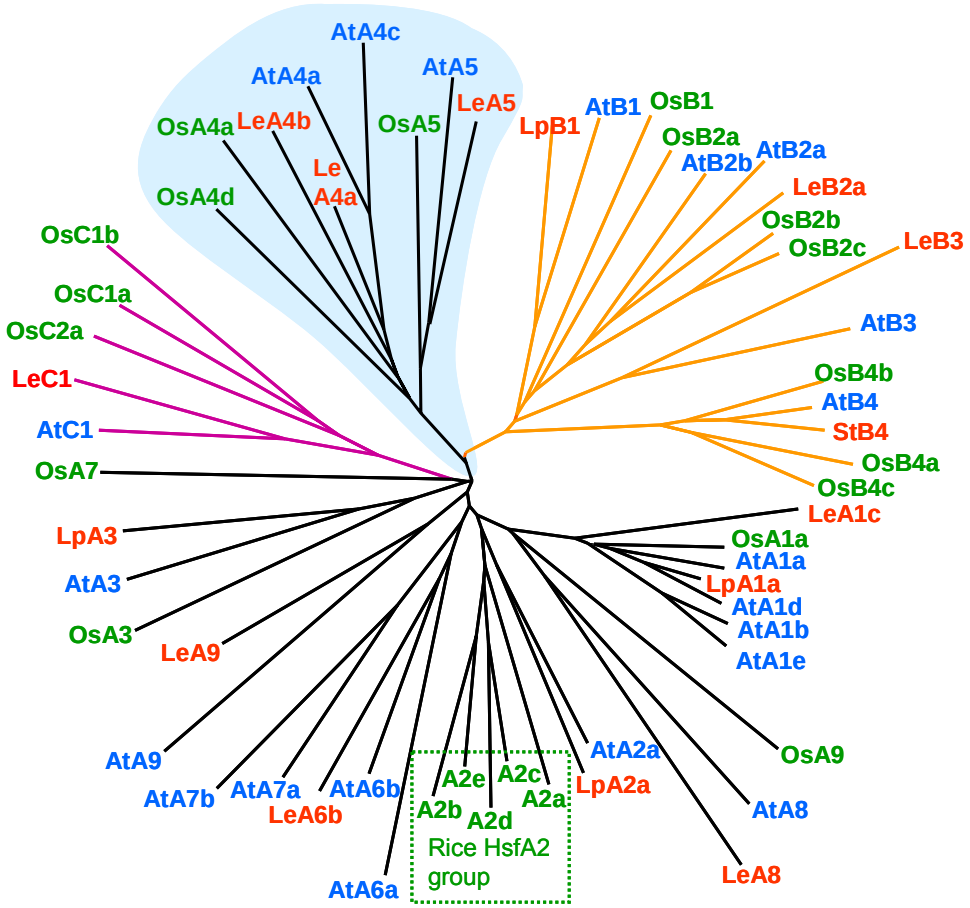


Figure 3.2 Phylogenetic tree based on amino acid residue sequences of the Hsf proteins from Arabidopsis, tomato, and rice. The tree was derived using Clustal 1-8_msw and Treeview software and is based on amino acid sequences of DBD and HR-A/B regions of Hsfs. Arabidopsis (At), tomato (Le) and rice (Os).

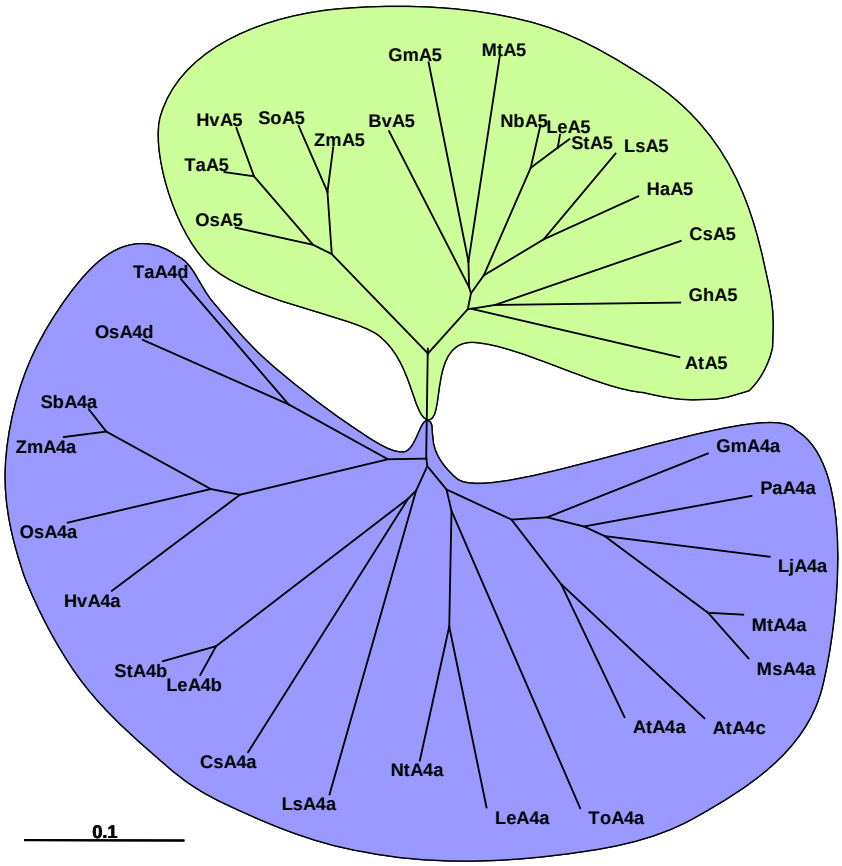


Figure 3.3 Phylogenetic tree of HsfA4 and HsfA5 from different plants.

The tree was derived using Clustal 1-8_msw and Treeview software and is based on amino acid sequences of DBD and HR-A/B regions of Hsfs. The numbers in bracket are the designated numbers for the retrieval of nucleotide sequences from the database (www.pubmed.com).

At *Arabidopsis thaliana* HsfA4a (At4g18880), HsfA4c (At5g45710), HsfA5 (At4g13980); **Bv**, *Beta vulgaris* HsfA5 (BQ488901); **Cs**, *Citrus sinensis* HsfA4a (DY270414), HsfA5 (DY300016); **Gh**, *Gossypium hirsutum* HsfA5 (DT548305); **Gm**, *Glyine max* HsfA4a (TC135284), HsfA5 (BM270993); **Ha**, *Helianthus annuus* HsfA5 (DY932755); **Hv**, *Hordeum vulgare* HsfA4a (TC42448), HsfA5 (TC151027); **Le**, *Lycopersicon esculentum* HsfA4a (BT014619), HsfA4b ((TC107140), HsfA5 (TC155271); **Lj**, *Lotus japonicus* HsfA4a (AP004978); **Ls**, *Lactuca sativa* HsfA4a (DY973605), HsfA5 (DY974369); **Ms**, *Medicago sativa* HsfA4a (AF494082); **Mt**, *Medicago truncatula* HsfA4a (TC79769), HsfA5 (TC79192); **Nb**, *Nicotiana benthamiana* HsfA5 (CK287755); **Nt**, *N. tabaccum* HsfA4a (AB014484); **Os**, *Oryza sativa japonica* HsfA4a (AP004879), HsfA4d (AC111015), HsfA5 (AP004999); **Pa**, *Phaseolus aureus* HsfA4a (AY052627); **Sb**, *Sorghum bicolor* HsfA4a (BM322601); **So**, *Saccharum officinarum* HsfA5 (CA264007); **St**, *Solanum tuberosum* HsfA4b (BG591987), HsfA5 (TC112724); **Ta**, *Triticum aestivum* HsfA4d (CV766704), HsfA5 (CJ655373); **To**, *Taraxacum officinarum* HsfA4a (DY824833); **Zm**, *Zea mays* HsfA4a (X92943), HsfA5 (EE174627).

3.3. Expression patterns of Hsfs A4 and A5 from tomato and Arabidopsis

3.3.1 Tomato

The numbers of ESTs detected in the databases indicate widespread expression of Hsfs A4 and A5 (S. Baniwal, Diploma thesis 2002). RT-PCR analyses using samples prepared from different tissues from tomato (Fig. 3.4 A, B) showed that the two A4 Hsfs (HsfA4a and HsfA4b) and HsfA5 have different expression patterns. HsfA5 mRNA was detectable in all the samples investigated. Similarly, HsfA4a mRNA was detectable in various samples *e.g.* cell culture, leaves, and flower buds. In contrast, HsfA4b mRNA was detectable only in cell culture and flower bud samples. HsfA4b mRNA level in flower bud was further studied in detail by using samples harvested from flower buds during different phases of maturation and dissecting them into different parts (gynoecia, anthers, sepals, and petals).

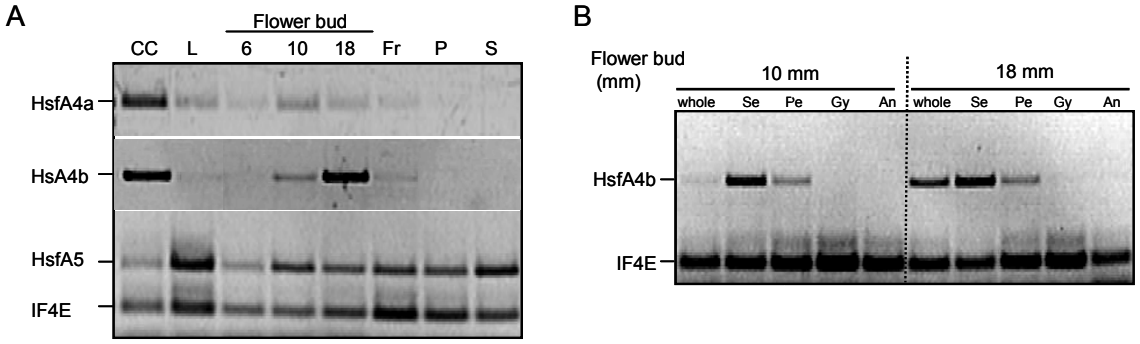


Figure 3.4 Expression analysis of tomato Hsfs A4a, A4b and A5.

(A) Primers specifically amplifying respective Hsf were used in RT-PCR with cDNA template synthesized from total RNA preparations from cell culture (CC); mature leaf (L); flower buds of approximate size (~length mm) 6, 10, and 18; very young fruits (~2 mm); pericarp (P); and developing seeds (S) obtained from 10 mm green fruits.

(B) Detailed analyses of HsfA4b expression in flower buds of length 10 mm and 18 mm respectively; Se, sepals; Pe, petals; Gy, gynoecia; An, anthers. Initiation factor 4E (IF4E) transcript was used as input control. Numbers of cycles used for PCR amplification were 35 for HsfA4a and 30 for other Hsfs or internal control.

The vertical length of the flower bud was taken as direct measure of the developmental stage. HsfA4b transcript was undetectable in samples obtained from flower buds of length less than 6 mm and was only detectable in samples obtained from flower buds of above 10 mm. Moreover, the transcript accumulation was detectable in the sepal and petal tissues, whereas samples from reproductive organs (gynoeceum and anthers) were essentially free of HsfA4b mRNA. These results showed that HsfA4b expression is restricted to mature sepal and petal.

3.2.2 Arabidopsis

Microarray analysis on the basis of newly generated AtGenExpress (see legend to Fig. 3.5) predicts that Arabidopsis Hsfs A4a, A4c and A5 mRNAs are indeed found in many tissues, albeit at very different levels (Fig. 3.5*A*). Usually, the levels of HsfA5 mRNA were markedly lower than those of Hsfs A4a and A4c. This contrasts to the results in tomato (Fig. 3.4) and the abundance of HsfA5 specific ESTs in the databases. Evidently, the situation in Arabidopsis is not typical. Elevated levels of HsfA4a mRNAs are found in samples prepared from leaves, especially senescent leaves and seedlings, whereas sample from pollen was enriched for Hsfs A4c and A5 mRNAs. Besides these developmental changes, expression of HsfA4a and, to a certain extend also of HsfA5, was stimulated by stress treatments (cold, salt, osmotic and heat stress), by UV-B as well as pathogen infection or elicitor treatments (Fig. 3.5*B, C*).

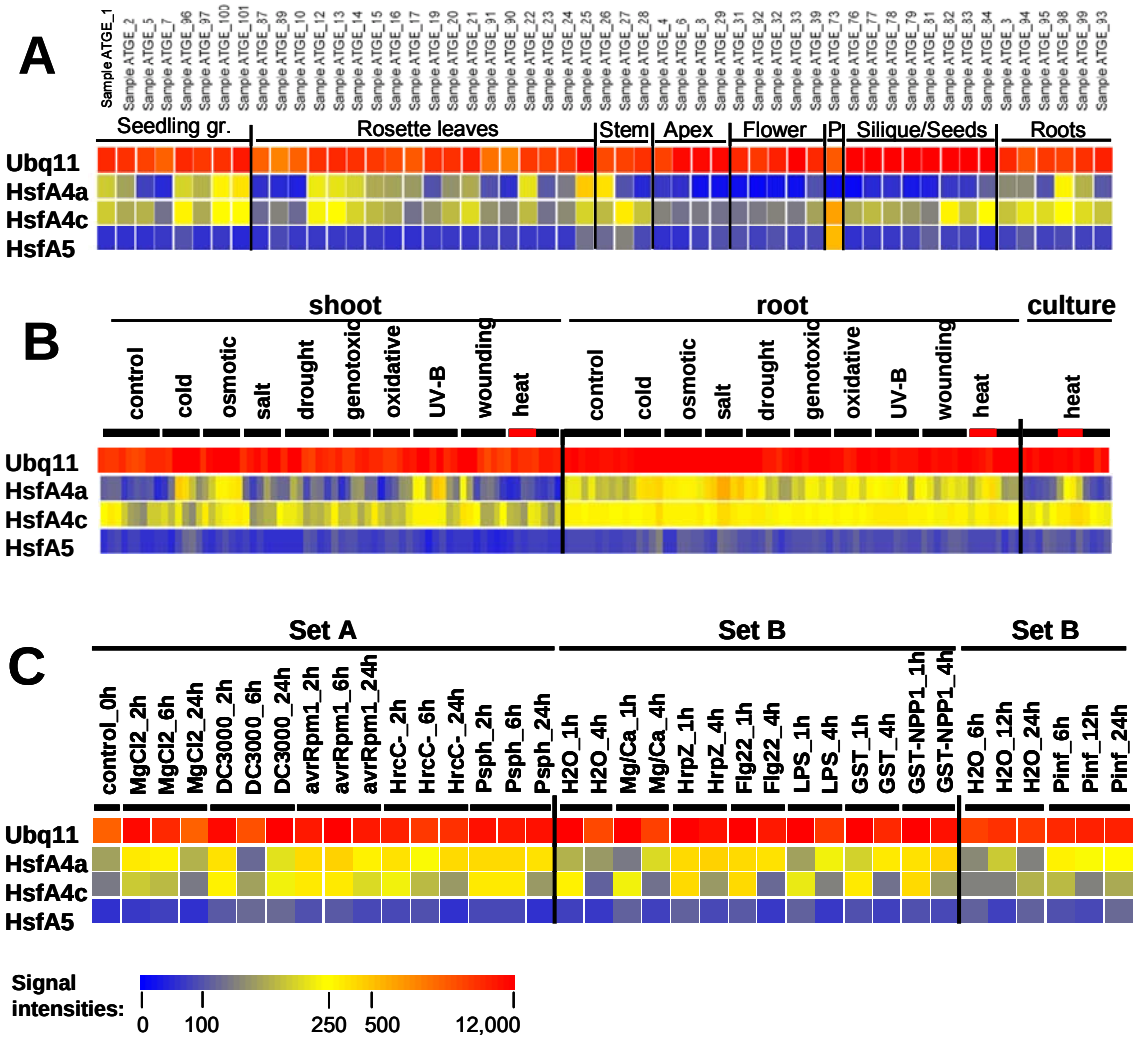


Figure 3.5 Expression analysis of Arabidopsis Hsfs A4a, A4c and A5 based on microarray data.

A, developmental series; ***B***, abiotic stress series; ***C***, pathogen infections, and elicitor treatments. The color code for the signal intensities is given at the bottom. Shown are the gcRMA normalized and averaged signal intensities (http://www.weigelworld.org/resources/microarray/AtGenExpress/) visualized as “heat maps” (with GeneSpring Version 7.2, Silicon Genetics) with re-transformed linear signal intensities for Hsfs A4a, A4c, and A5. Ubiquitin 11 mRNA levels were used as constitutive expression control. (Data assembled by Pascal von koskull Döring).

3.4 Transactivation properties of tomato Hsfs A4b and A5 in tobacco protoplasts

Since the early publication of Treuter *et al*. (1993) tobacco protoplasts were established as excellent system to study the potential of Hsf to activate transcription of Hsp genes (Lyck *et al*. 1997; Scharf *et al*. 1998, Döring *et al*. 2000). This test was considerably enriched by including the evaluation of chromatin embedded Hsp genes of tobacco, Hsf proteins once synthesized bind to their target sequences in the promoters of Hsp encoding genes and activate their transcription. The resulting expression can be assessed by western blotting using specific antisera *e.g.* for Hsp17-CI (Mishra *et al*. 2001, Port *et al*. 2004). Although seemingly quite different, both tests gave comparable results.

As a starting point for HsfA4 and HsfA5 characterization, tomato HsfA4b and HsfA5 were cloned in suitable plant expression vectors and analyzed in such assays (Fig. 3.6). Hsfs A1a, A2 and A3 were also incorporated into the tests to compare their transcriptional activities. A fixed amount of tobacco protoplasts were transformed with the expression plasmids for individual Hsf and a GUS reporter plasmid and the GUS activities were measured (Fig. 3.6*B*). The GUS activity observed in a sample transformed with the reporter plasmid only represents the basal level expression due to the endogenous Hsf cocktail present in tobacco protoplasts (marked with dotted line). GUS activities above this level correspond to the transactivation potential of the transiently expressed Hsf. Expression of all Hsfs except

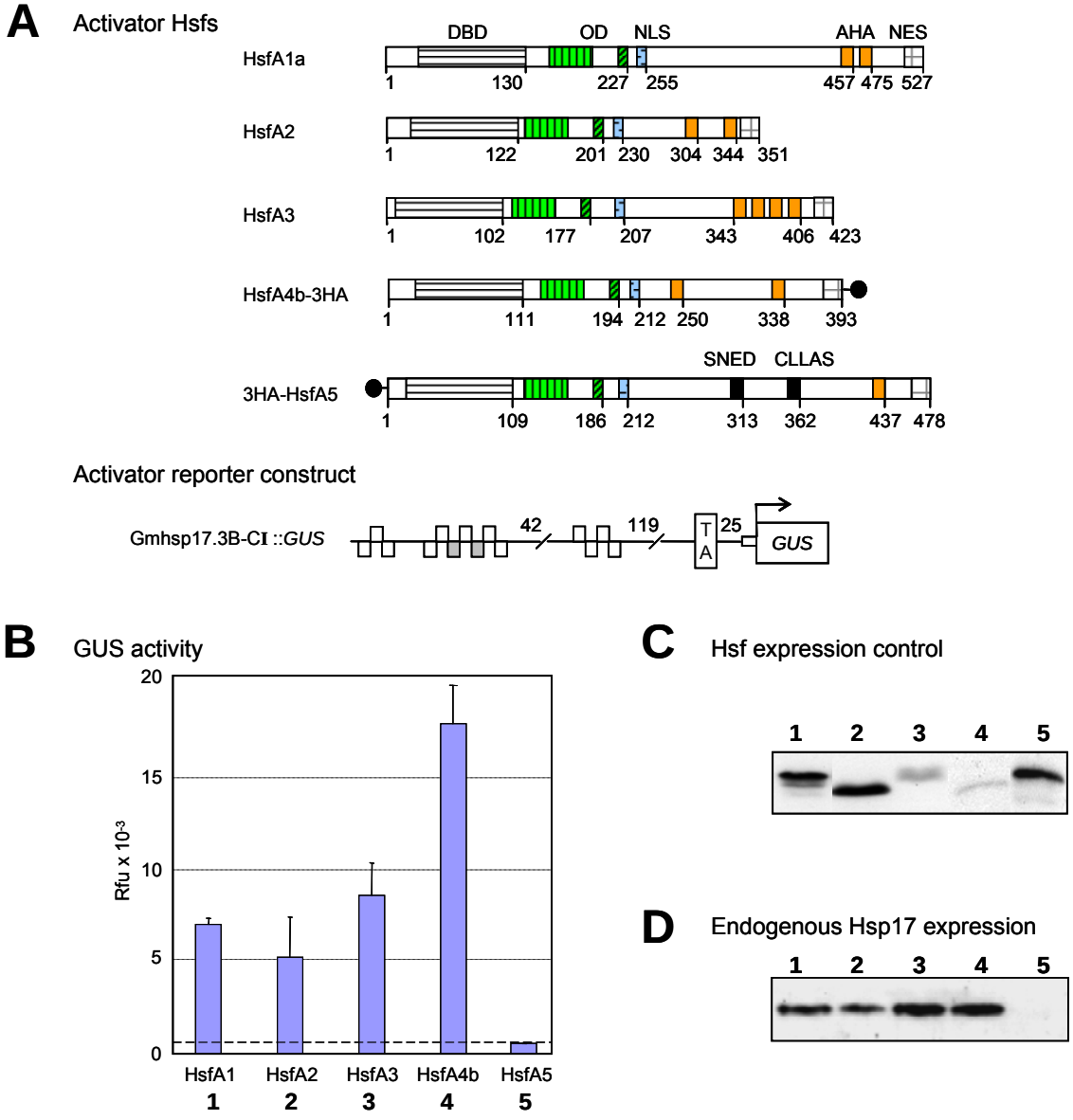


Figure 3.6 Assessment of Hsf activator function by their transient expression in tobacco mesophyll protoplasts.

A) *Block diagrams* representing the basic architecture of activators and the activator reporter construct used. Numbers refer to the amino acid residues. Hsfs A4b and A5 were tagged at their C/N-termini respectively with triple HA to facilitate their detection (black dots).

Activator reporter containing the soybean Gmhsp17.3B-CI promoter fragment fused to GUS gene. The fragment consists of the indicated combinations of heat stress elements (HSE, for details see Nover et al. 2001) and TATA box (TA). Numbers indicate the distance in base pairs

B, C, and D) *Protein blots* detecting the expression levels of Hsfs **(C)** and Hsp17-CI **(D)** by using α-8HN (HsfA1a), α-pep6 (HsfA2), α-HsfA3, α-HA (Hsfs A4b, HsfA5) and α-pep17 (Hsp17-CI).

GUS activity						
Reporter	Endog.	AtHsfA1a	AtHsfA2	AtHsfA4a	AtHsfA4c	LeHsfA4b
pHsp17.4-CI::GUS	980	10,860	18,070	4,020	13,370	17,170
pHsp18.1-CI::GUS	650	14,270	17,290	1,970	16,630	18,500
pHsp70b-CI ::GUS	320	8,390	15,060	485	3,320	17,270
pHsp101.3 ::GUS	1,860	7,560	8,360	2,570	8,970	16,620
pApx2 ::GUS	1,170	8,060	13,370	3,720	11,860	17,010

Table 3.2 Survey of transactivation potential of Hsfs using transient expression reporter assays.

Different reporters carrying ~1 kb upstream sequences of the indicated genes as promotor for GUS expression. GUS activities are given as Rfu (see Materials and methods).

Endog., endogenous activity without additional activators used as background.

HsfA5, stimulated the expression of reporter gene. In agreement with the GUS activities, expression of endogenous Hsp17 genes was also readily detectable from all samples except from HsfA5 (Fig. 3.6*D*). Inactivity of HsfA5 was not simply because of low protein level as its expression was many folds higher than that of HsfA4b which was also 3HA-tagged for detection (Fig. 3.6*C*). In addition, it was found that similar results were obtained by using several reporter constructs carrying promotor region of different Hsp genes (Table 3.2). These results indicate that Hsfs A4 of tomato and Arabidopsis are good activators, whereas HsfA5 is inactive with all reporters tested (data not incorporated in Table 3.2)

3.5 Potential of tomato Hsfs A4b and A5 to replace yeast Hsf (ScHsf1)

The first Hsf encoding gene cloned and analyzed was Hsf1 of *S. cerevisiae* (Sorger and Pelham, 1988). Furthermore, it was found that Hsf1 function is essential for yeast cells not only to survive stress conditions but also for growth at normal temperatures. The yeast Hsf1 can be replaced by heterologous Hsf, *e.g.* tomato Hsfs A1a, A2, and A3 (Boscheinen *et al*. 1997; Bharti *et al*. 2000). Similarly, human Hsf1 and Hsf2 were also examined for their capacity to replace yeast Hsf1 (Liu *et al*. 1997). In contrast to wild type Hsf2, Hsf1 failed to compliment. However, derivatives of Hsf1 with short C-terminal deletions, could efficiently substitute for yeast Hsf1. All these studies underlined the conservation of heat stress response and the absolute requirement of each Hsf to activate target gene expression; *e.g.* mutations affecting Hsf activator function also affected its ability to support survival and growth in yeast.

Tomato HsfA4b and HsfA5 together with previously characterized HsfA2 were tested in Hsf replacement test in yeast. All transformed yeast strains with the corresponding Hsf expression plasmids grew normally on non-selective media, *i.e.* under conditions where the yeast Hsf1 is still present. By treatment with FOA (5-fluoroorotic acid) the URA-3 gene containing plasmid encoding the yeast Hsf1 is eliminated, and the resulting strain was now dependant on the heterologous Hsf. The results show that similar to HsfA2, HsfA4b supported yeast growth whereas HsfA5 did not (Fig. 3.7*A*). However, yeast strains expressing HsfA4b or HsfA2 failed to support growth at 37^{o}C, while cells expressing ScHsf1 did show normal growth even at 37^{o}C (Fig. 3.7*B*). Taken together, these observations reveal that unlike other class-A

Hsfs tested in yeast (Boscheinen *et al*. 1997; Bharti *et al*. 2000) there is something special with HsfA5.

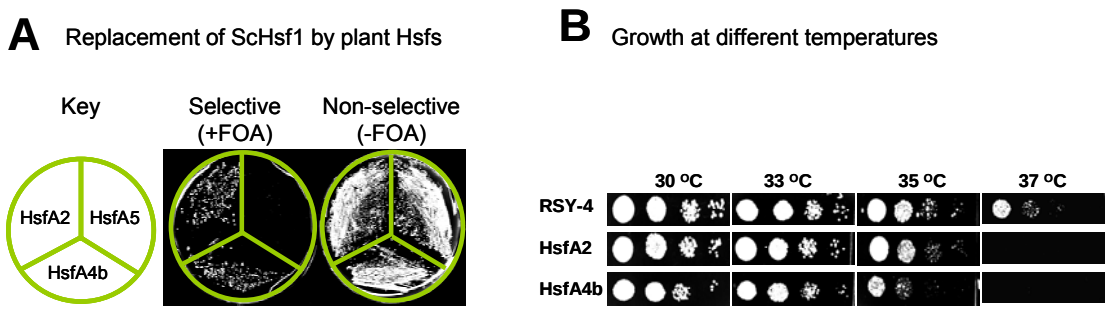


Figure 3.7 Assessment of Hsf transactivator function in yeast cells.

(A) *Functional replacement of yeast Hsf1 by plant Hsfs using RSY4 strain*- Selection of yeast transformants carrying plasmids encoding the indicated Hsf by growing on non-selective media (-W, -H, -U, -FOA) at 30^0C. Growth of the strains on selective media (-W, -H, +U, +FOA), appearance of colonies indicate the replacement of ScHsf1 by the plant Hsf.

(B) Temperature-stress tolerance of different yeast strains carrying the indicated Hsfs rich growth media.

3.6 DNA binding potential of Hsfs A1a, A4b, and A5

Sequence analysis of HsfA5 gave no immediate clue for an understanding of its inactivity. Thus, the potential DNA binding was examined. To this aim, repressor reporter assay was used that allows *in-vivo* assessment of DNA binding activity independent of the activator function of Hsfs (Treuter *et al*. 1993). The repressor reporter contains multiple Hsf binding sites immediately downstream of the TATA box of the constitutively active CaMV 35S promoter (Fig. 3.8*A*). Recognition of these HSE elements by Hsf will affect the progression of "charged" RNA polymerase-II or will interfere with assembly of the transcription complex. In this assay diminishing of *GUS* gene transcription is an additive effect of the Hsf expression level and its DNA binding affinity. Indeed, Hsfs A1a, A4b and A5 strongly reduced the detectable GUS activity (Fig. 3.8*B,* samples 2 to 8). Reference points are sample 1 showing GUS activity in the absence of transiently expressed Hsfs and sample 9 expressing a DNA binding mutant form of HsfA5. Since detection of all the Hsf proteins in this experiment was carried out by using HA tag, their expression levels could be directly compared with one another. By collectively considering the GUS activities and intensities from protein blot, the relative strengths for DNA binding of the three Hsfs could be compared with each another. HsfA4b has the highest and HsfA1a and HsfA5 have lower DNA binding affinities. But the differences are not very striking. So it could be concluded that the inactive behavior of HsfA5 in reporter assays was not due to poor DNA binding potential.

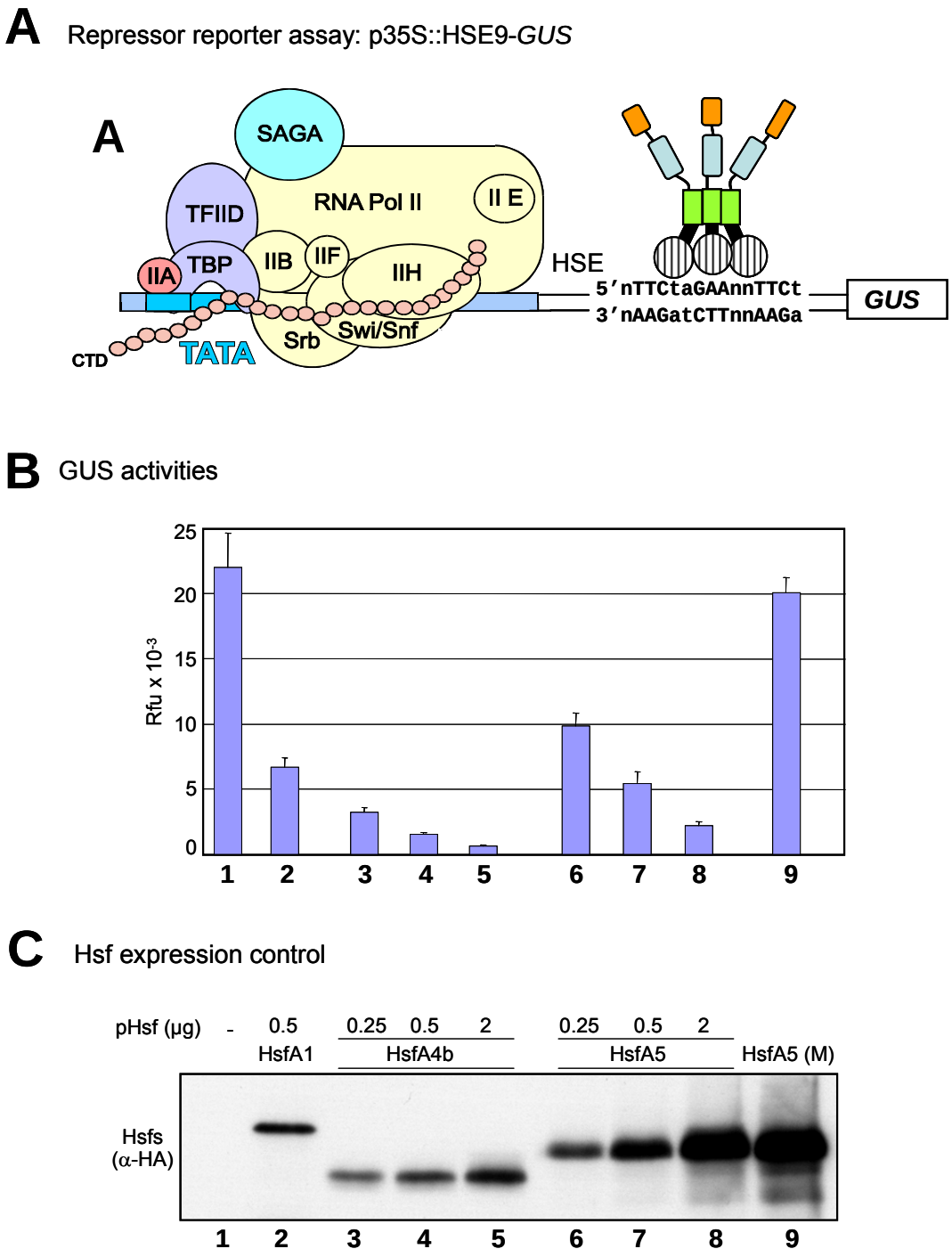


Figure 3.8 Use of the repressor reporter assay to test for DNA binding affinity.

(A) Representation of the concept of repressor reporter which contain array of nine consecutive HSE immediately downstream of TATA-box; (TA). Hsf binding to HSE would interfere with the progression of charged RNA polymerase II **(B)** GUS activities after transformation of protoplasts with the repressor reporter and the indicated amounts of Hsf expression plasmids. **(C)** Hsf expression controls, protein blot as Hsf expression by α–HA.

3.7 Domain swapping experiments with the functional modules of HsfA5

Based on amino acid sequence homology with other class A Hsfs, all the functional modules required for Hsf function as transcriptional activator could be identified for HsfA5. Yet no detectable transactivation potential could be observed in any of the test systems. To investigate this discrepancy in more detail, various hybrid constructs with structural parts from other potent transcriptional activators *e.g.* HsfA2, HsfA4b, and VP16 were generated.

3.7.1 Fusion proteins with C-terminal domain of other activator Hsfs

The first group of constructs contained the N-terminal parts of HsfA5 (Fig. 3.9*B* marked red in the block diagram) combined with C-terminal parts of strong activator HsfA2 (constructs 4, 5, 6) and HsfA4b (construct 2) (Fig 3.9). As positive controls plasmids encoding wild type HsfA2 and HsfA4b were also tested. All Hsf proteins accumulated to readily detectable levels and GUS activities observed for all of them were high (see respective panels in Fig. 3.9). These results confirm that the DBD of HsfA5 is fully functional and intact.

3.7.2 Fusion proteins with domain swapped with VP16 activation domain

For the second group of constructs, we used the AD of VP16, which is commonly used as a model for acidic activators. VP16 has no preferred binding to specific DNA sequences alone but recognizes target sequences of Herpes simplex virus (HSV) immediate early genes in conjunction with Oct-1 and HCF-1 (Liu *et al*. 1999). Similar to the AHA-domain of Hsfs, the activation domain of VP16 (VP16-AD) possess a typical pattern of aromatic aa residues. The activator potential is due to its interaction with many components of the transcription machinery as well as with chromatin modifying complexes.

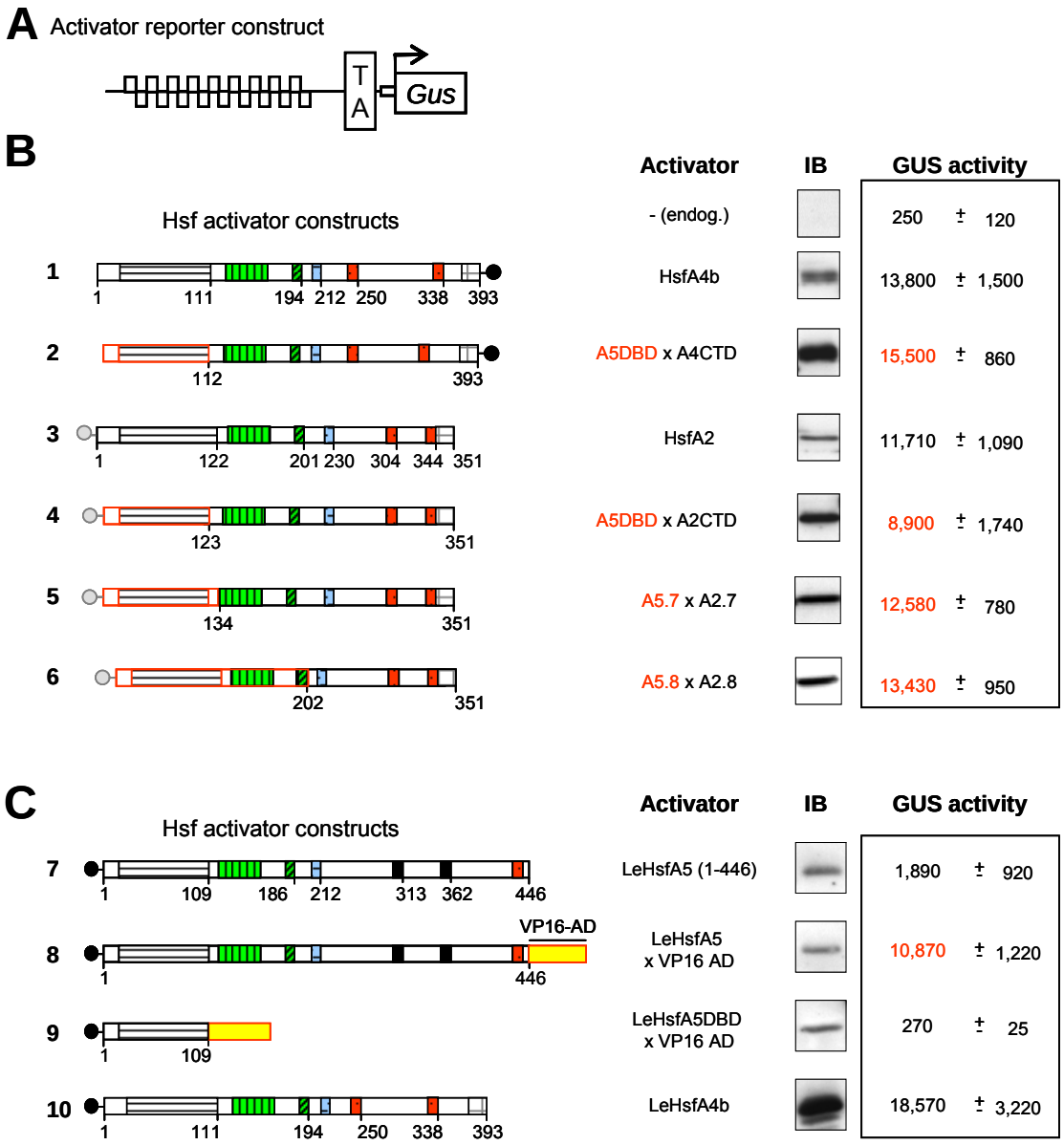


Figure 3.19 Hybrid Hsf composed of N-terminal parts from HsfA5 fused to the activator domains of other Hsfs or to VP16 (411-490).

(A) Hsf dependent HSE9-*GUS* reporter co-transformed with Hsfs.

(B and C) Domain architecture of Hsf proteins, GUS activities and Immuno-blot (IB) analysis of the Hsfs used, hybrid Hsfs (constructs 2, 4, 5, and 6), the N-terminal part derived from HsfA5 have been drawn in red color (containing DBD and/or OD) and the numbers represent aminio acid residues. Black and grey dots are 3HA- and Myc- tags respectively. Constructs 8 and 9 contain the C-terminal 81 aa from VP16 (called VP16-AD)

In the hybrid constructs the VP16-AD was fused either immediately adjacent to the DBD of HsfA5 or at the extreme C-terminus immediately adjacent to the AHA motif (Fig. 3.9*C* constructs 8, 9). As control, full length HsfA4b (construct 10) and HsfA5ΔC446 (construct 7) were used. All Hsfs accumulated to similar levels (see expression controls). As expected, HsfA4b was highly active and HsfA5ΔC446 was inactive. However, the fusion protein of HsfA5 with VP16-AD at the C-terminus (construct 8) strongly activated the reporter construct. This result rules out the argument, that the CTD of HsfA5 contains a repressor function that restricts the access of AHA motif to the components of the transcriptional machinery. Interestingly, fusion construct 9 bearing VP16-AD immediately adjacent to HsfA5 DBD was completely inactive. It is most likely due to the lacking oligomerization domain as monomeric Hsfs were shown to be inefficient in DNA binding (Boscheinen *et al*. 1997).

3.7.3 Fusion proteins with Gal4 DNA binding domain

In the third group of fusion proteins the functionality of the HsfA5 CTD was examined by generating hybrid constructs with the DBD of the yeast transcription factor Gal4 (1-147 amino acid residues) (Fig. 3.10). In the reporter assay, GalBDxHsfA5CTD (fusion construct 3) expression mildly stimulated the reporter as compared to the negative control with the Gal4DBD alone (construct 1) and Gal4BDx- HsfA1aCTD (construct 2). Most significantly, the stimulation was lost by replacing the crucial amino acid residues FW in the AHA motif by AA. The results clearly indicate the functionality of the HsfA5CTD. However it should be noted that this is a specialized test condition where Gal4BD might influence additional properties of HsfA5 CTD which otherwise exist in its wild-type context.

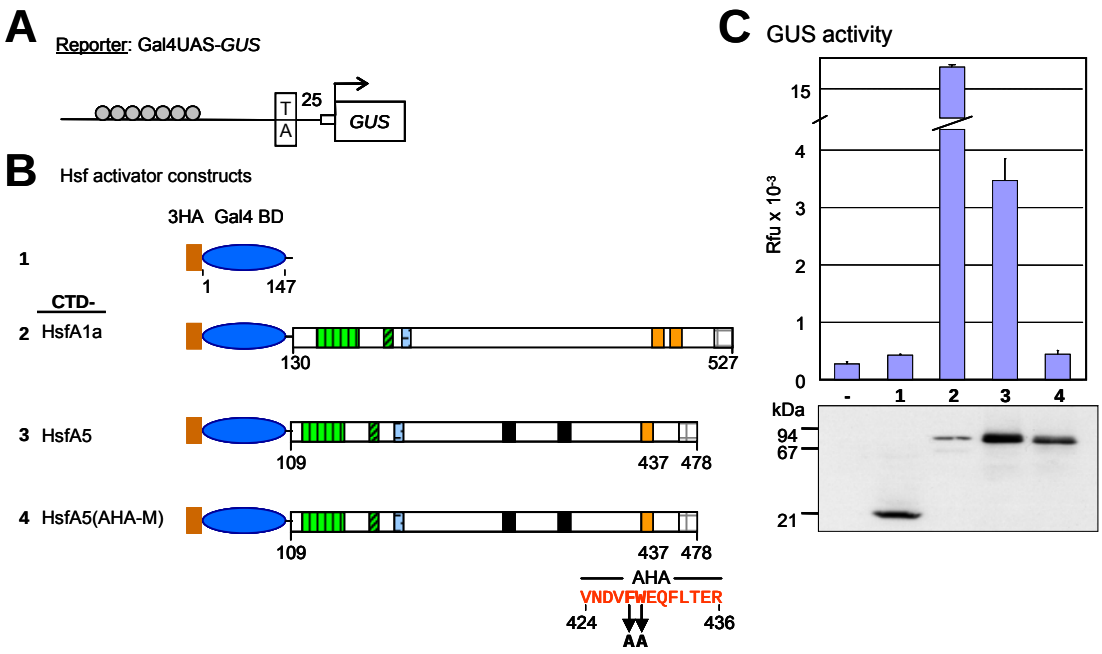


Figure 3.20 HsfA5 harbors a functional albeit weak activation domain.

(A) Schematic representation of Gal4UAS-*GUS* promotor containing the multiple binding sites for Gal4 transcription factor (grey dots).

(B) Block diagrams representing the structural modules of various proteins used in GUS assay.

(C) GUS activities and protein expression controls (*upper* and *lower panels*) of the Gal4BD and other fusion proteins. 3HA tag was fused at the N-termini of these constructs for detection.

3.8 Activator/repressor relationship between HsfA4b and HsfA5

The close structural relations and the surprising differences in the activator function of Hsfs A4 and A5 prompted us to investigate a possible interaction of both Hsfs. In Fig. 3.11*B*, the GUS activity observed in a sample transformed with the reporter plasmid only represents the basal level expression due to the endogenous Hsf cocktail present in tobacco protoplasts (marked with dotted line). GUS activities above this level correspond to the transactivation potential of the transiently expressed Hsfs. Expression of HsfA4b alone (sample 1) showed nearly 40 fold stimulation of reporter gene expression as compared to the basal level, whereas expression of HsfA5 showed no activity (sample 8). In agreement with the GUS activities, expression of endogenous Hsp17 genes was high in sample 1 but not detectable in sample 8 (Fig. 3.11*C*). Protein blot analysis (Hsf expression control, Fig. 3.11*B*) showed that both HA-tagged Hsfs accumulated to detectable levels. Usually, the level of HsfA5 was higher than that of HsfA4b, ruling out the simple explanation that low expression of HsfA5 was the cause for its lack of activity. It could thus be concluded that HsfA4b is a strong activator, whereas HsfA5 has no activator potential on either plasmid borne or chromatin-embedded reporters.

The contrasting behavior of the two Hsfs in reporter assays were surprising as both share very similar basic structural features, particularly of their DNA binding domains and of their C-terminal domains containing the activator motifs (see block diagram in Fig. 3.11 and Table 4.1). To examine any functional interaction between them, I co-transformed tobacco

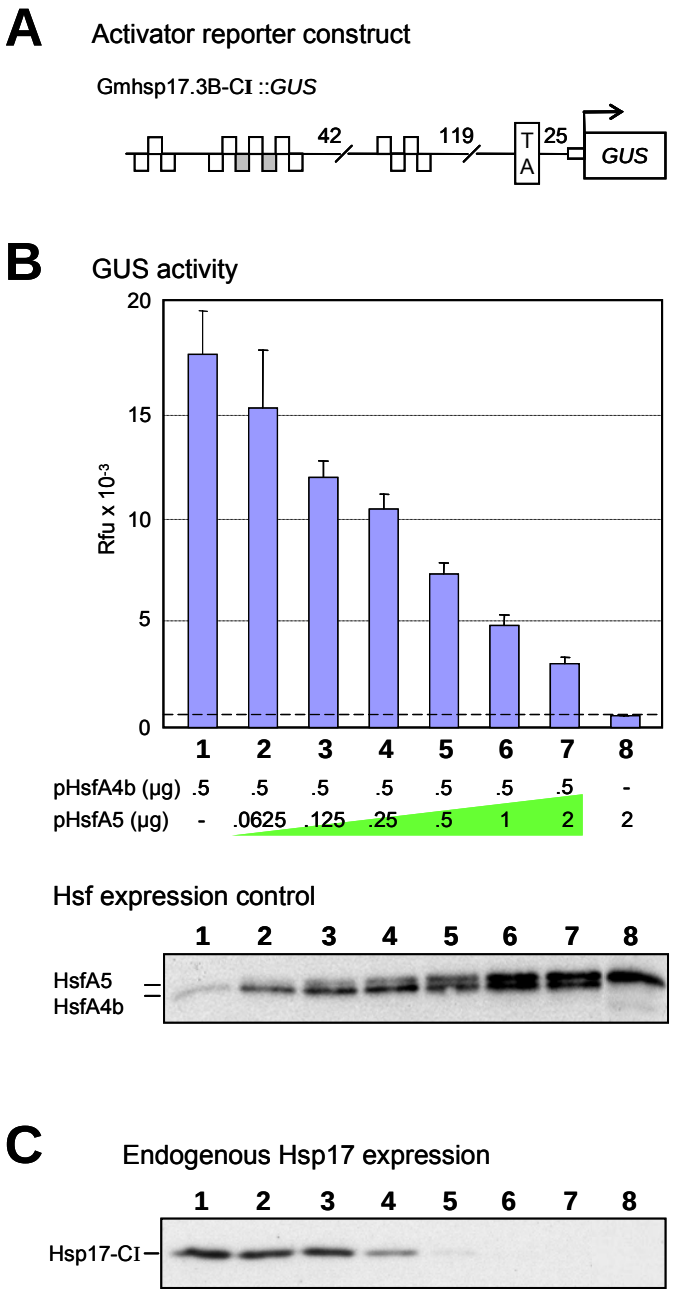


Figure 3.9 HsfA5 as repressor of HsfA4b activator function in tobacco protoplasts.
(Figure obtained from Baniwal *et al.* 2007)

A) *Reporter containing the soybean Gmhsp17.3B-CI promoter fragment fused to GUS gene.* The fragment consists of the indicated combinations of heat stress elements (HSE, for details see Nover et al. 2001) and TATA box (TA). Numbers indicate the distance in base pairs

B and C), *Effects of HsfA5 on the activator potential of HsfA4b*. GUS reporter activity (Rfu, Relative fluorescence units) in samples transformed with the indicated amounts of Hsf expression plasmids (µg/20,000 protoplasts) and protein blot analyses showing expression of 3HA-tagged Hsfs A4b and A5 **(B)**. **C)** Expression of the endogenous Hsp17 encoding genes detected by antisera for Hsp17-CI.

protoplasts with a constant amount of HsfA4b and increasing amounts of HsfA5 expression plasmids (Fig. 3.11*B*, samples 2 to 7). Two effects were observed: (i) The stability of HsfA4b increased as a result of HsfA5 co-expression. (ii) In contrast to the increased HsfA4b accumulation, the GUS reporter activity decreased in direct correlation with increasing HsfA5 expression (see Hsf expression control and GUS activity in samples 2 to 7 respectively in Fig. 3.11*B*). Similar effects of HsfA5 on HsfA4b activity were observed on the endogenous Hsp17 level as reporter (Fig. 3.11*C*, samples 1 to 7). Interestingly, the repressor effect of HsfA5 was more pronounced on the chromatin-embedded Hsp17 than on the plasmid borne GUS reporter gene (compare results with samples 1 and 5 in Figs. 3.11*B* vs. *C*).

3.9 HsfA5 affects activity of no other class A Hsf

Next, I examined whether the repressor effect of HsfA5 is specific for HsfA4b or HsfA5 functions as a general repressor of Hsf activity, *e.g.* by competing for DNA binding sites. To this aim, I co-expressed HsfA5 together with other well characterized members of the tomato Hsf family, *i.e.* Hsfs A1, A2 and A3 (Fig. 3.12). All three Hsfs are potent transcriptional activators (samples 9, 11, 13). However, the activity of none of them was repressed in the presence of HsfA5, but was rather increased (Fig. 3.12*B*, samples 10, 12, 14). I conclude that the HsfA5 function as repressor is highly specific for HsfA4b and does not simply result from competition for Hsf binding sites.

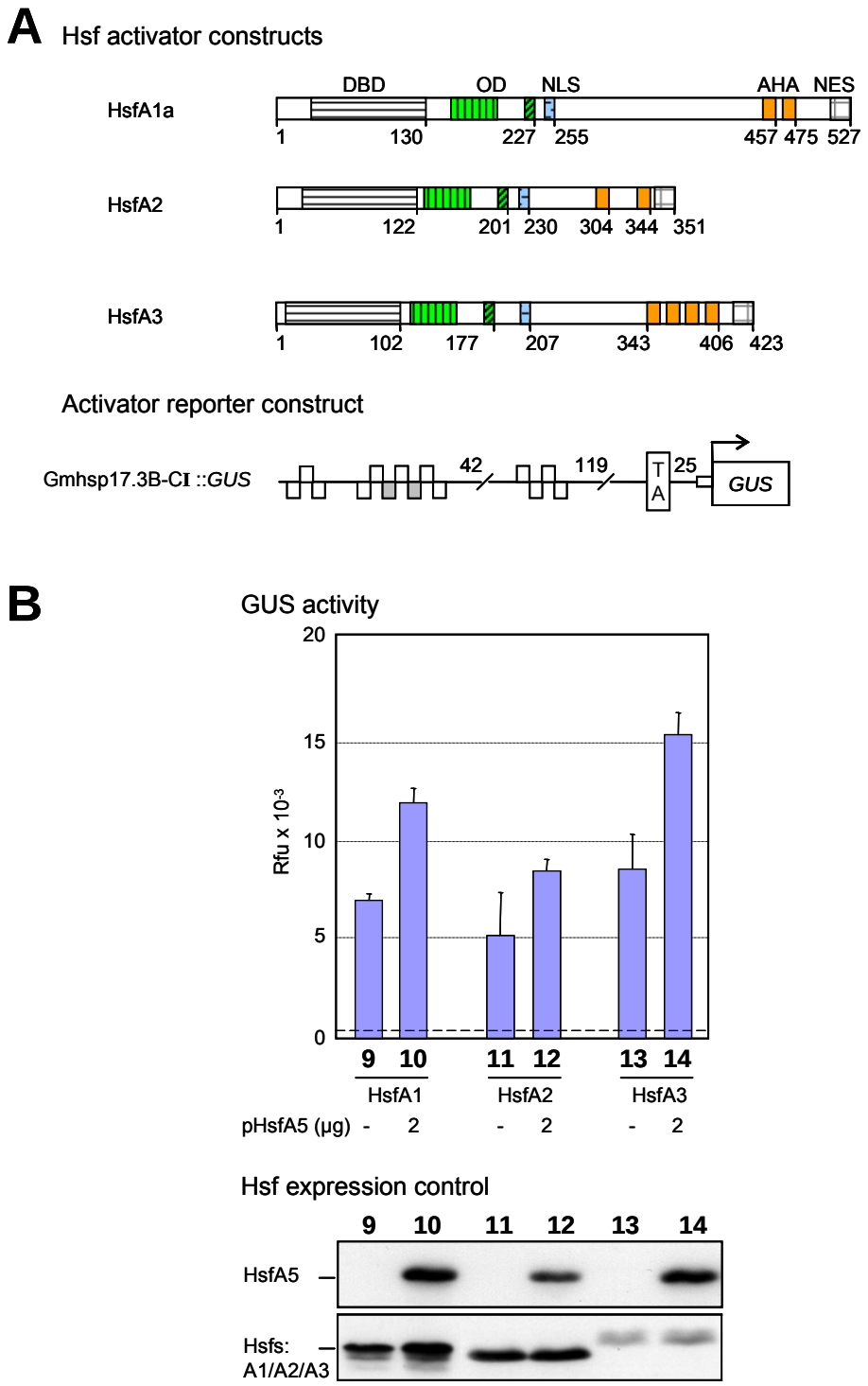


Figure 3.10 Effects of HsfA5 on the activator potential of Hsfs A1, A2 and A3.

(Figure obtained from Baniwal *et al.* 2007)

(A) Block diagrams representing the reporter construct and the Hsfs.

(B) GUS activities and **(C)** Expression of Hsfs. Anti-HA for HsfA5 (*upper panel*) and Hsf specific antisera for Hsfs A1, A2 and A3 (*lower panel*) were used.

3.10 HsfA5 does not affect the heat stress response of tobacco protoplasts

As an additional proof for the selectivity of HsfA5 repressor function, I expressed HsfA5 and examined the hs response of the tobacco protoplasts following stress temperature treatment as indicated in Fig. 3.13*B*. The accumulation of Hsp17 in the mock transformed protoplasts (Fig. 3.13*C*) reflects the hs-induced activity of the tobacco Hsf system. It was completely blocked by the expression of a dominant negative form of tomato HsfA1a, *i.e.* HsfA1a∆C394, which competes for DNA binding but has no activator function. In contrast to this, expression of HsfA5 had no detectable effect. Evidently, the hs-induction in these mesophyll protoplasts completely depends on the activity of A1-type but not on A4-type Hsfs. Furthermore, HsfA5 does not act as a general repressor. These results nicely confirm the fundamental differences between Hsfs A1, A2, and A3 on one hand and Hsfs A4/A5 on the other.

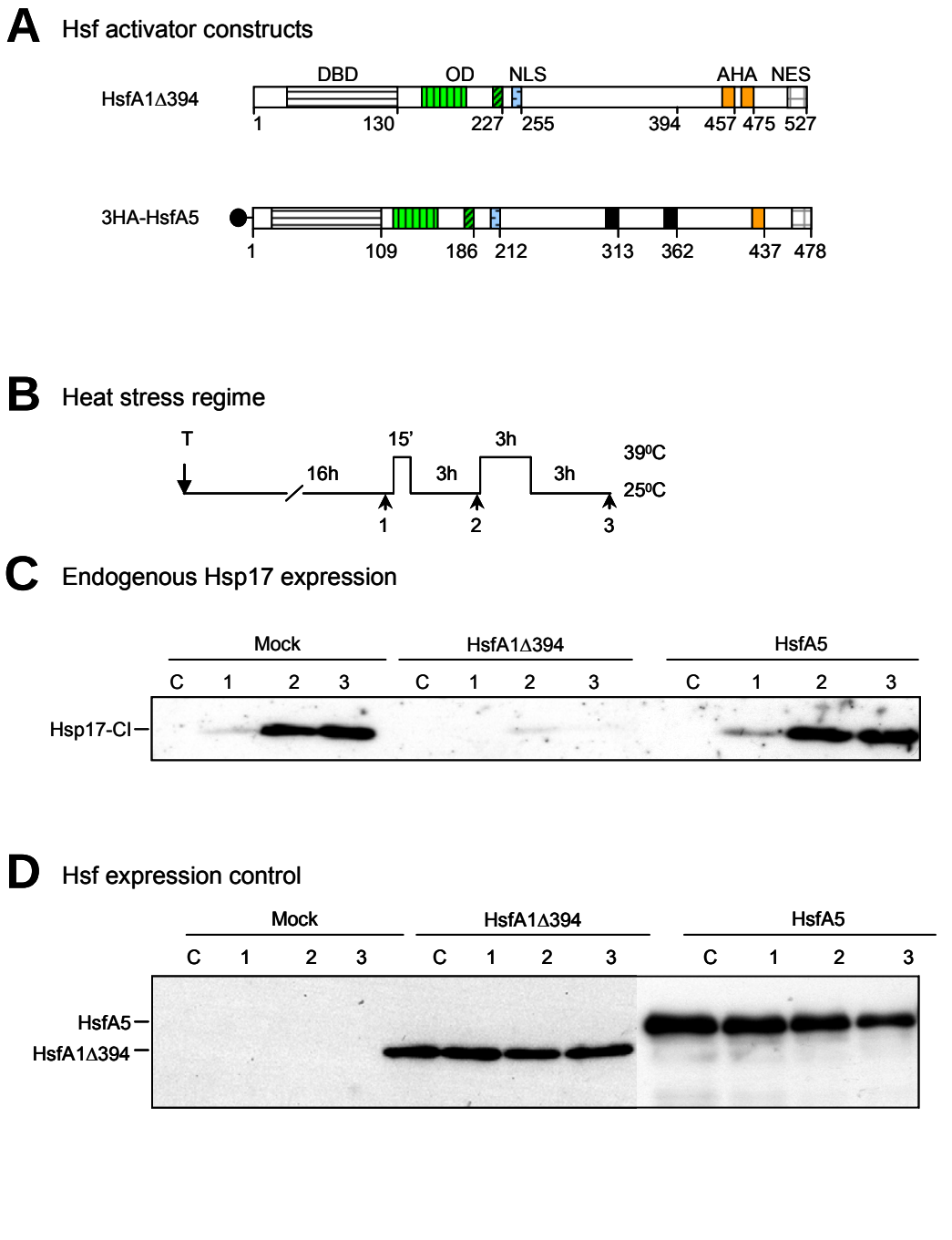


Figure 3.13 HsfA5 does not inhibit the heat stress (HS) induced expression of Hsps in tobacco protoplasts.

(Figure modified from Baniwal *et al.* 2007)

(A) Block diagrams of Hsf used in the experiment, the arrow in HsfA1 block diagrams indicates the position of stop codon resulting in the expression of aa 1 to 394.

(B) Pictograph showing the heat stress regimen. Samples C (control), and 1, 2, 3 were harvested at the indicated time points. T, indicates transformation of protoplasts with empty vector (mock) or with expression plasmids encoding HsfA1ΔC394 and HsfA5 respectively.

(C) protein blot analysis of the whole cell extracts from indicated samples by using α-HsfA1, α-HA or α-hsp17.

3.11 Repression of HsfA4b activity is mediated through the oligomerization domain of HsfA5

In order to define the structural elements of HsfA5 required for the repression of HsfA4b activity, I prepared a series of deletion forms (Fig. 3.14*A*, constructs b to e) and a DNA binding mutant form (construct f) and tested them with respect to their repressor activity in transient reporter assays (Fig. 4*B*, *C*). As expected from the previous results (Fig. 3.6), none of the HsfA5 mutant forms showed any activity on their own (data not shown), but as long as they contained the oligomerization domain (OD) they effectively repressed HsfA4b activity (Fig. 3.14*B*, samples 4, 7, 8). Note that the smallest HsfA5 fragment with full repressor activity contained only the OD (construct e). Evidently, the oligomerization domain of HsfA5 is necessary and sufficient to exert the repressor effect on HsfA4b. In support of this, HsfA5 fragments lacking the OD (constructs c and d) did not repress HsfA4b activity (Fig. 3.14*B*, samples 5, 6).

DNA binding and transcriptional activation by Hsfs are strongly dependent on their oligomerization state. Hsf deletion mutants lacking their OD are poor in DNA binding and activator function (Boscheinen *et al.* 1997). The repressor reporter assay described above allowed us to test, if the DNA binding activity of HsfA4b could be affected by co-expression with HsfA5 or its mutant forms (Fig. 3.14*C*). The effects observed can be summarized as follows: (i) As shown before (Fig. 3.8), HsfA4b blocked the *GUS* expression (Fig. 3.14*C*, sample 2), and co-expression of HsfA5 enhanced this effect (sample 3). This enhancement is mainly due to the marked increase of the HsfA4b level in the presence of HsfA5 (Fig. 3.14*D*, sample 3) (ii) In

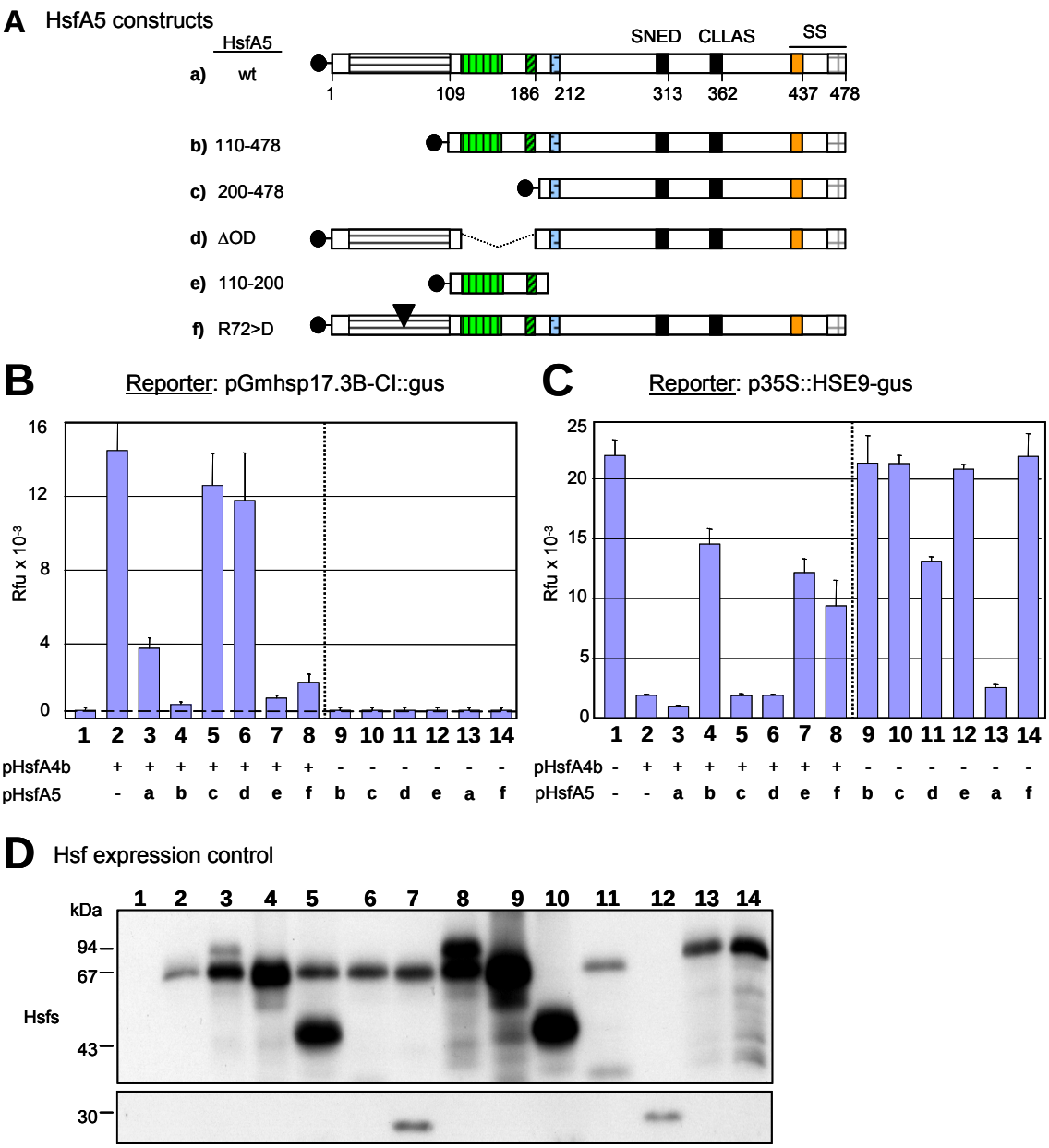


Figure 3.14 Structural requirements for the repressor function of HsfA5.

(Figure obtained from Baniwal *et al.* 2007)

(A) Block diagrams representing HsfA5 and its mutant forms used for co-transformation with HsfA4b. Wild-type HsfA5 (a), different truncation forms (b to e) and a DBD mutant in which the invariant Arginine aa residue at position 72 in the HTH motif was mutated to Aspartate (R72>D). Black dots mark triple HA-tag at the N-terminus used for detection and OD is abbreviated form of oligomerization domain (HR-A/B).

(B) GUS expression levels in samples containing HsfA4b alone and its combination with indicated forms of HsfA5 (sample1 and samples 2 to 8 respectively). Samples 9 to 14 contain the indicated HsfA5 form alone.

(C) Repressor reporter assay with sample composition as in B.

(D) Hsf expression controls for samples 1 to 14.

combinations of HsfA4b with HsfA5 mutant forms containing the OD (samples 4, 7, 8), *GUS* expression was much less diminished than in sample 3. Evidently, interaction with the truncated forms of HsfA5 affected the DNA binding affinity of HsfA4b. (iii) As expected, mutant forms of HsfA5 lacking the OD had no influence on the HsfA4b mediated block of *GUS* expression (Fig. 3.14*C*, samples 5 and 6). Taken together, these results imply that interaction of HsfA5 with HsfA4b may disturb the oligomerization state of the latter and thereby drastically decreases its DNA binding capacity and function as transcriptional activator.

3.12 Presence of compatible interactive interfaces is mandatory for HsfA5 mediated repression

The structural prerequisites of HsfA4b, *i.e.* functional DNA binding and oligomerization domains as well as the C-terminal activator domain with AHA motifs, are basically similar with those defined for HsfA1a and HsfA2 (Döring *et al*. 2000; Bharti *et al*. 2004). Therefore, I wanted to know, whether the repressor effect of HsfA5 could also act on HsfA1a, provided the OD regions are compatible for interaction. To this aim, I tested HsfA1a wt and a hybrid form containing the oligomerization domain of HsfA4b (Fig. 3.15*A*). As predictable from the results shown in Fig. 1*C*, the expression levels of Hsp17 in samples with HsfA1a wt were not affected by the presence of HsfA5 (Fig. 3.15*B*, samples 1, 2, 3). However, the high activity of the hybrid form observed in sample 4 was completely abolished in the presence of the HsfA5 fragment (Fig. 3.15*B*, samples 5 and 6). Interestingly, inactivation of the hybrid HsfA1a caused by HsfA5 fragment was accompanied by a marked stabilization of the protein (see protein blot analyses in Fig. 3.15*C*). This effect reminds of earlier observations in yeast and mammalian cells suggesting inactive transcription factors to be more stable because of proteasome activities being intricately connected with active transcription complexes (Muratani and Tansey, 2003).

The physical interaction between HsfA5 fragment and different HsfA1a forms were confirmed by GST-fusion protein pull-down technique (Fig. 3.15*D*). GST alone was used as negative control as it did not pull down any of the Hsfs under the assay conditions. The bait *i.e.* GST-HsfA5 (aa 110-200) pulled down HsfA4b as well as the hybrid form of HsfA1a but not wt HsfA1a

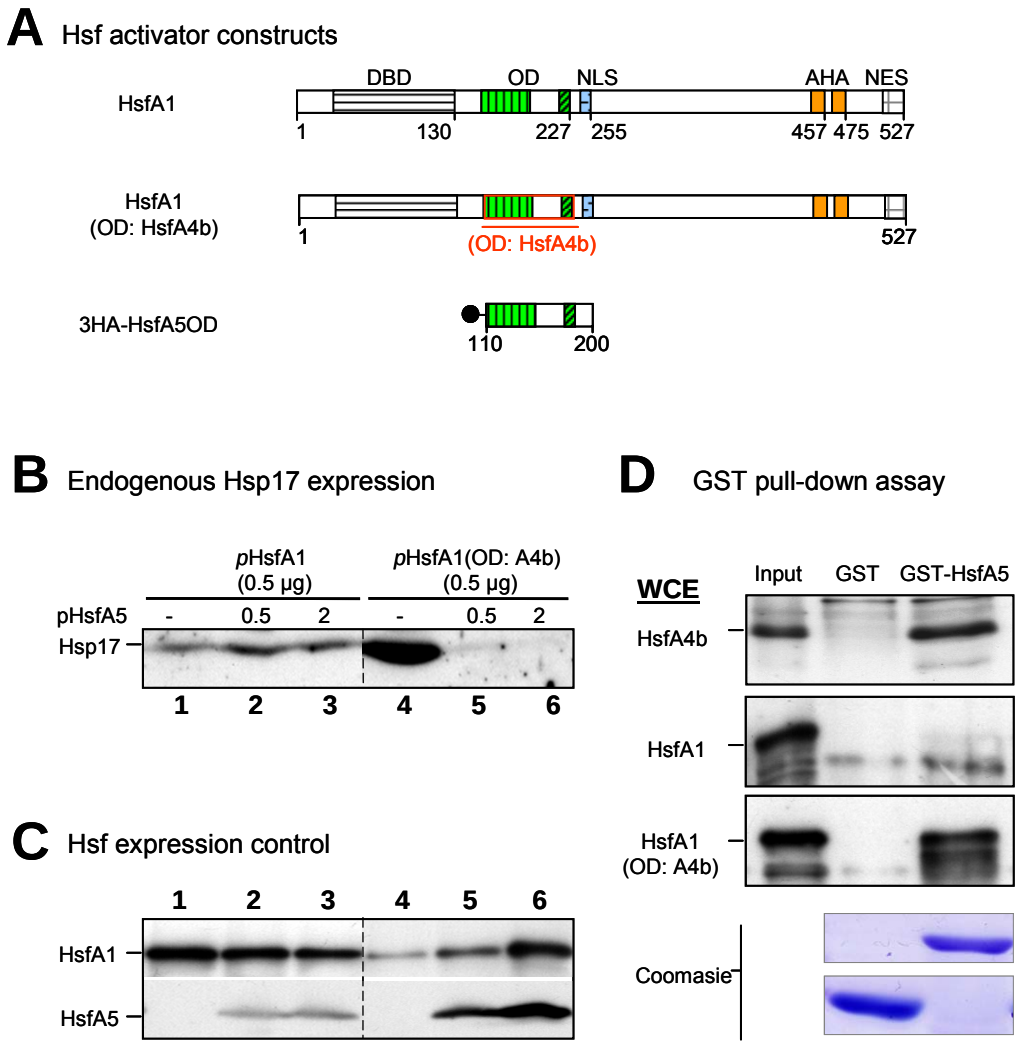


Figure 3.15 Oligomerization domain of HsfA4b is sufficient to make HsfA1 sensitive to the repressor effect of HsfA5.

(Figure modified from Baniwal *et al.* 2007)

(A) Block diagrams of HsfA1 and HsfA1 carrying the HR-A/B region (OD) of HsfA4b and HsfA5OD fragment used as repressor.

(B) Expression of endog. Hsp17 reporter in protoplast samples transformed with the indicated Hsf expression plasmids (*upper panel*) and expression of Hsfs using serum against HsfA1 and HA (*lower panel*).

(C) GST-fusion protein pull down assay using purified GST and GST-HsfA5 (fragment) fusion proteins as baits to pull down prey proteins (indicated on the left side of each protein blot). Whole cell extracts (WCE) were prepared from *E.coli.* expressing the prey proteins: HsfA4b-Strep, HsfA1 and HsfA1(OD:HsfA4b). α-Strep detected HsfA4b-Strep and α-Hsf1 detected HsfA1 wt as well as its mutant form.

indicating that the presence of HsfA4b oligomerization domain in HsfA1a was necessary and sufficient to allow stable interaction with HsfA5.

The necessity of compatible interactive interface was further corroborated by the weakening of repressor effect mediated by HsfA5 carrying the oligomerization domain of HsfA1a. Increasing amount of expression plasmids for HsfA5 or HsfA5(OD:HsfA1a) were co-transformed with a constant amount of HsfA4b encoding plasmid in tobacco protoplasts. The repressor effect of HsfA5(OD:HsfA1a) was found to be markedly lower as compared to the wt HsfA5 (Fig. 3.16, compare GUS activities and Hsp-17 expression levels between samples 1 to 6 *vs* 12 to 17). Moreover there was no mutual stabilization of HsfA4b and HsfA5(OD:HsfA1a) in contrast to its combination with wt HsfA5 (see the Hsf expression control for samples1 to 6 and 12 to17). The results from this assay fall in-line with my earlier observations (previous section) and thereby it may be concluded that for HsfA5 the repressor effect is primarily defined and executed by the oligomerization domain.

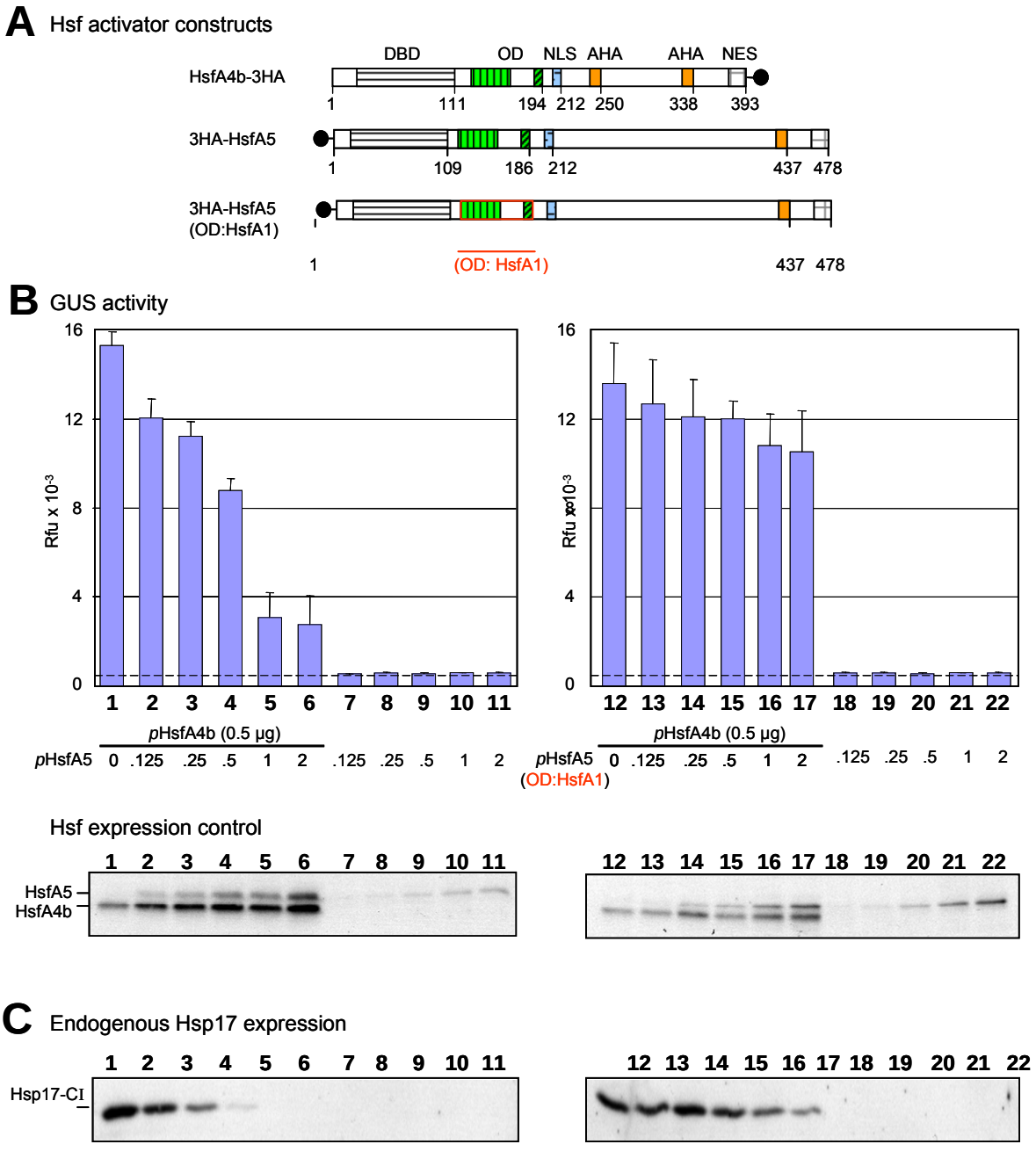


Figure 3.16 Repression of HsfA4b by wild type and oligomerization domain mutant forms of HsfA5.

Each part of the figure i.e. A, B, C depicts: HsfA5 form used (block diagram on top), reporter activities (GUS, plotted as graph and endog. Hsp17, as protein blot with thick boundary) and expression controls for Hsfs, in samples transformed with the indicated combination of plasmids encoding respective Hsfs in tobacco mesophyll protoplasts.

3.13 The activator/repressor relationship of Hsfs A4 and A5 is also found for Arabidopsis Hsfs

In Arabidopsis two genes encode A4-type Hsfs, namely AtHsfA4a and AtHsfA4c (At4g18880 and At5g45710) and one gene encodes AtHsfA5 (At4g13980). The basic structure of these Hsfs is very similar to their tomato homologues (block diagrams in Fig. 3.17*A* and Table 4.1). Because of the relatively low activator potential of the two Arabidopsis Hsfs A4a and A4c as compared to tomato HsfA4b, I used a high affinity activator reporter (pHSE9::*GUS*) for these tests (Fig. 3.17*B*). The results were very similar to those for tomato Hsfs, *i.e.* AtHsfA5 effectively repressed the activities of both AtHsfs A4a and A4c (Fig. 3.17*B*, samples 3 and 6) and in the repressor reporter assay co-expression with AtHsfA5(R77D) relieved the expression block by AtHsfs A4a and A4c (Fig. 3.17*C*, samples 3 and 5).

To further examine the specificity for AtHsfA5 mediated repression, other class A Hsfs of Arabidopsis, *e.g.* Hsfs A1a, A1b, A2 A3 etc were tested by co-expression with AtHsfA5 (Fig. 3.17*E*). None of the tested AtHsf was affected by co-expression with AtHSfA5. These results are in full agreement with the conclusion derived from tomato Hsf system *i.e.* HsfA5 specifically and effectively repressed A4 type Hsf.

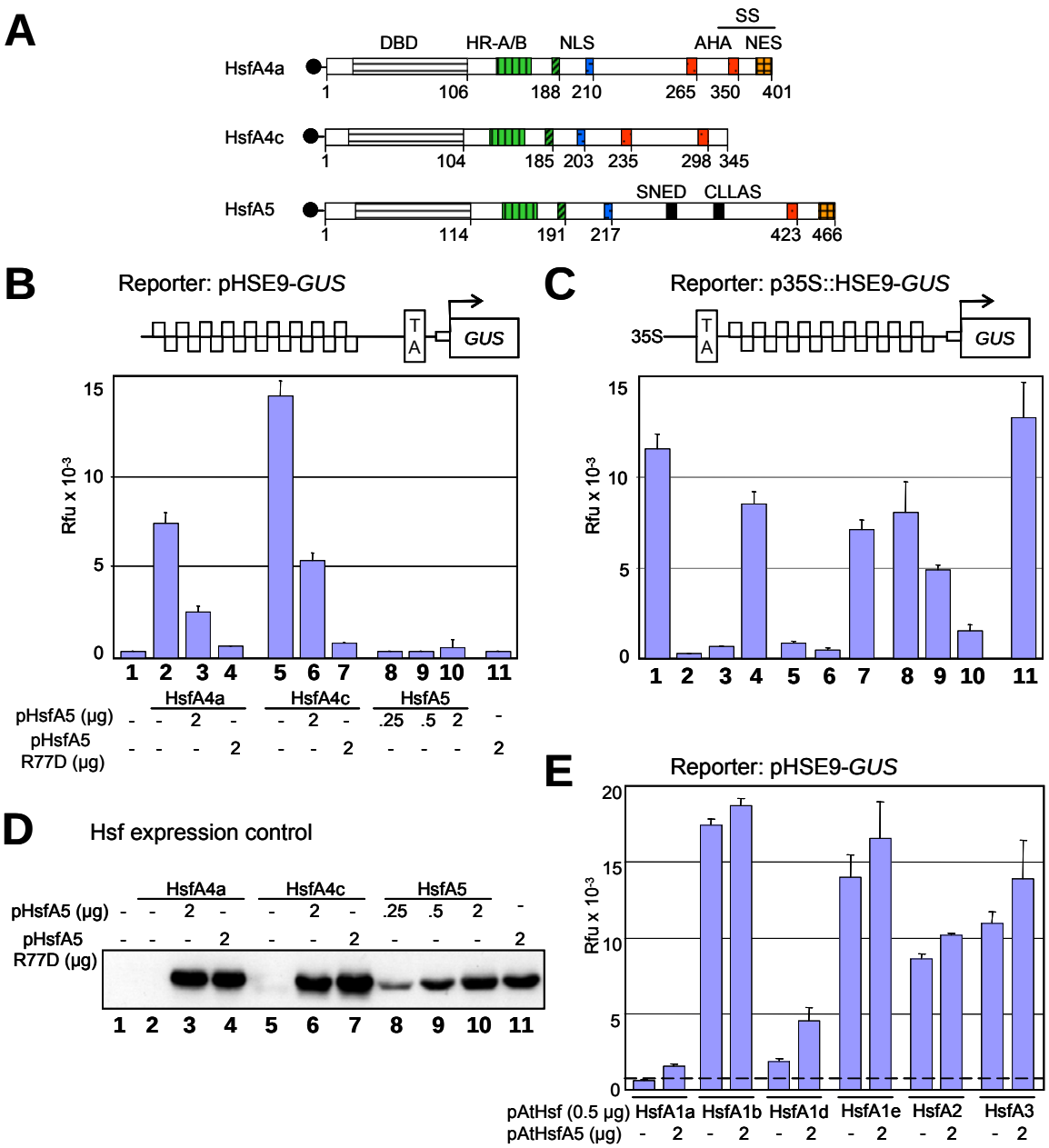


Figure 3.17 Arabidopsis HsfA5 specifically represses transcriptional potential of AtHsfs A4a and A4c.

(Figure obtained from Baniwal *et al.* 2007)

(A) Block diagrams depicting basic structures of the AtHsfs.

(B and C) Tobacco protoplasts were transformed with the indicated plasmids, i.e. 0.5 µg of Hsfs A4a and A4c and 2 µg of HsfA5 expression plasmids either with activator reporter pHSE9:*GUS* (B) or with the repressor reporter (C).

(D) Protein blot as Hsf expression control by using α-HA.

(E) Different class A1 members, HsfA2 and HsfA3 were expressed alone or in combination with AtHsfA5 in tobacco protoplasts.

3.14 Physical interaction between HsfA4/A5 from Arabidopsis and tomato

The interpretation of the repressor effect of HsfA5 on the activator function of HsfA4 implicates an unprecedented specificity of recognition among the A4/A5 group of Hsfs, which evidently excludes other members of the class A Hsfs. To support this conclusion I utilized several independent approaches using Hsfs from both Arabidopsis and tomato. First, the protein interactions among Arabidopsis Hsfs were investigated using yeast two hybrid system. Various yeast two hybrid constructs were derived with Hsfs A1a, A4a, A4c and A5 in bait and Hsfs A4c and A5 in prey positions (Fig. 3.18*D*). All yeast strains resulting from transformation with the indicated sets of plasmids grew normally on non-selective medium (-WL), but on selective medium (-WLH and –WLH +10 mM 3-AT), only strains with strong protein interactions produced colonies. These are exclusively the strains with heterodimeric protein interactions within the A4/A5 group (see rows 3, 5, 8, 9). As expected, no interactions were found with the Gal4-DBD alone (nos. 1 and 6) or with HsfA1a in bait position (rows 2, 7). Surprisingly, even the homodimeric interactions within the A4/A5 group were too weak to be detected in this system. The results clearly indicate that in the yeast two hybrid test, which is based on dimeric protein interactions, formation of heterooligomers between Hsfs A4 and A5 was much preferred as compared to homooligomers.

In addition to AtHsfA1a, other Arabidopsis Hsfs were also examined for their potential to interact with AtHsfs A4a, A4c, and A5 using yeast two hybrid assay. None of the Hsfs was found to show interaction with the members of the HsfA4/A5 subgroup (M. Port, PhD thesis 2006).

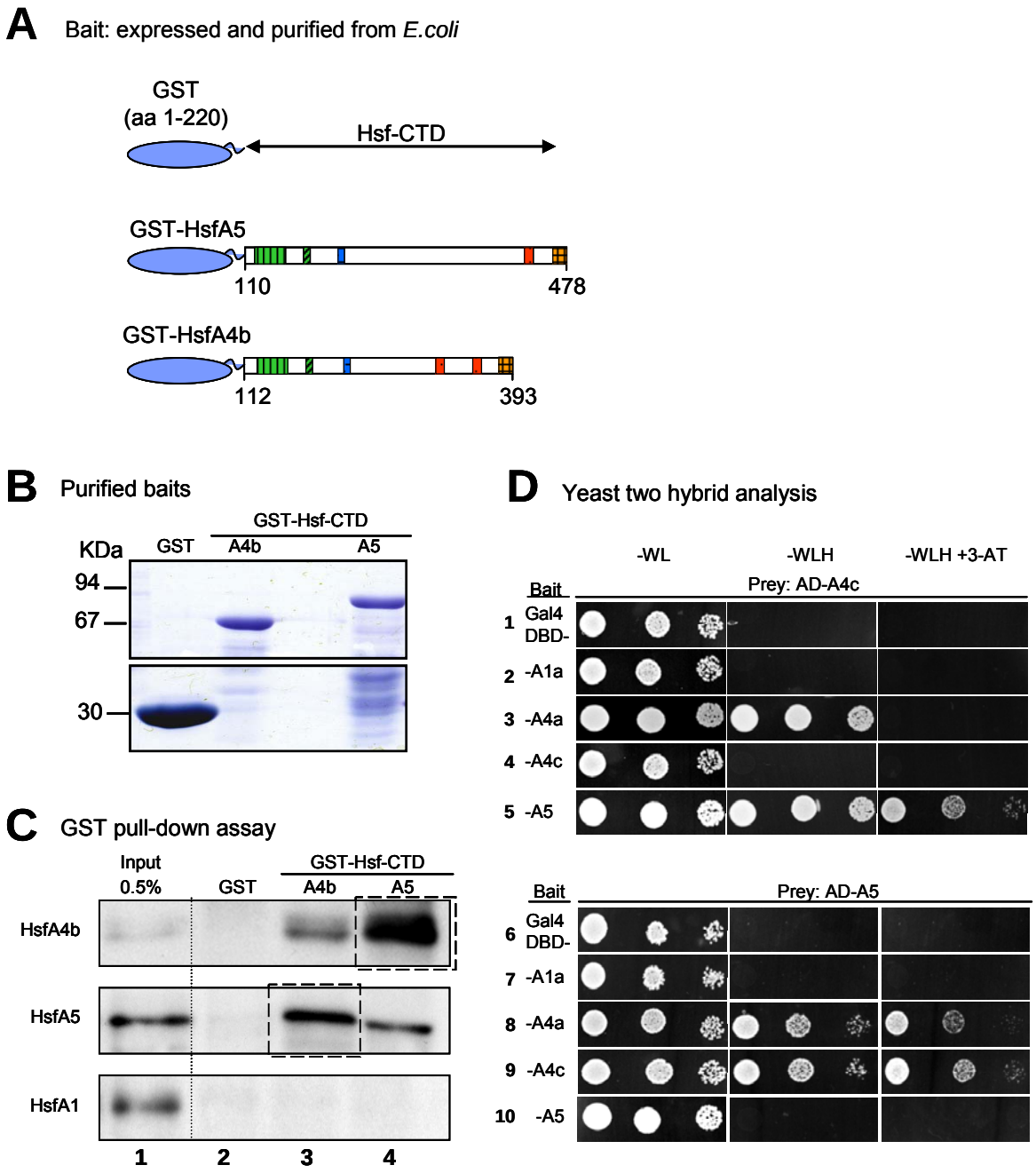


Figure 3.18 Pull-down assay and yeast two hybrid analysis to detect interactions among tomato and Arabidopsis Hsfs.

(Figure modified from Baniwal *et al.* 2007)

A and B) Block diagrams to represent the baits used and purified baits after SDS-PAGE and coomassie staining.

C) Immunoblot analysis of the material following pull-down, using α-HA (HsfA5), α-Strep (HsfA4b), and α-8HN (HsfA1)**.**

D) Yeast two hybrid test using AtHsfs A4c and A5 as prey and AtHsfs A1a, A4a, A4c and A5 as baits (see Materials and Methods for bait and prey details). Abbreviation used: AD and BD are activation and binding domains respectively of yeast transcription factor Gal4; W, L, H are aa selection markers Tryptophan, Leucine, Histidine respectively; 3-AT is 3-aminotriazole inhibitor for Histidine biosynthesis used at 10 mM concentration.

Second, pull-down assays with tomato Hsfs A4b and A5 fused to glutathione-S-transferase as baits and different whole cell extracts expressing Hsfs A1, A4b and A5 were used to assess physical interaction among them (Fig. 3.18*A*, *B, C*). As baits, in addition to GST-HsfA4b and GST-HsfA5, same amount of GST protein was in parallel used as background control. Following pull-down with same amounts of Hsf-bait proteins the signals detected from the heterologous combinations (boxed) were clearly much stronger than those from the homologous combinations; as control, no detectable pull-down of any of the three Hsfs was detectable with GST alone as bait. Moreover, no pull-down of HsfA1a was detectable with any of the three baits used. The results and conclusion obtained from these two techniques were in perfect correlation with each other *i.e.* HsfA4 and HsfA5 have higher tendency to form heterooligomers with each other than to form homooligomers among themselves.

3.15 Dynamics of the intracellular distribution of HsfA4b and HsfA5

It is well known from investigations with tomato and Arabidopsis Hsfs that many of them contain signals for nuclear import (NLS) and export (NES) and that changes of the intracellular localization influences their activity (Lyck *et al*. 1997; Heerklotz *et al*. 2001; Kotak *et al*. 2004). To study the localization of HsfA4b and HsfA5, the corresponding GFP fusion proteins were expressed in tobacco mesophyll protoplasts (Fig. 3.19*A*). The GFP-HsfA4b was detected in the nucleus, whereas GFP-HsfA5 was predominantly in the cytoplasm (Fig. 3.19*B*, samples 1 and 3 respectively). However, after addition of Leptomycin B (LMB), an inhibitor of the nuclear export receptor, GFP-HsfA5 strongly accumulated in the nucleus (Fig. 3.19*B*, sample 4). This result clarifies that, similar to the tomato HsfA2 (Heerklotz *et al*. 2001), the NES function of HsfA5 dominates its NLS function. Although localized mainly in the cytoplasm, HsfA5 shuttles between cytoplasm and nucleus. Application of heat stress had no detectable effect on the intracellular distribution of GFP-HsfA5 (Fig. 3.19*C*).

3.15.1 Identification of nuclear export signal of HsfA5

A typical nuclear export signal represents a short hydrophobic sequence with a typical pattern of Leucine residues, LXXLXL (*e.g.* tomato HsfA2, Heerklotz *et al*. 2001). The C-terminal of HsfA5 (~150 aa) contain such putative NES motifs including the C-terminal peptide VEHLTL*. To validate the relevance of these motifs for nuclear export function, two mutants were constructed (Fig. 3.19*A*). These mutant Hsfs contain GFP at their N-termini fused either to HsfA5 aa 1-330 or aa 1-440 called GFP-HsfA5Δ330 and GFP-HsfA5Δ440 respectively. GFP-HsfA5Δ440 that lacked the C-terminal NES motif had (continue page 61)

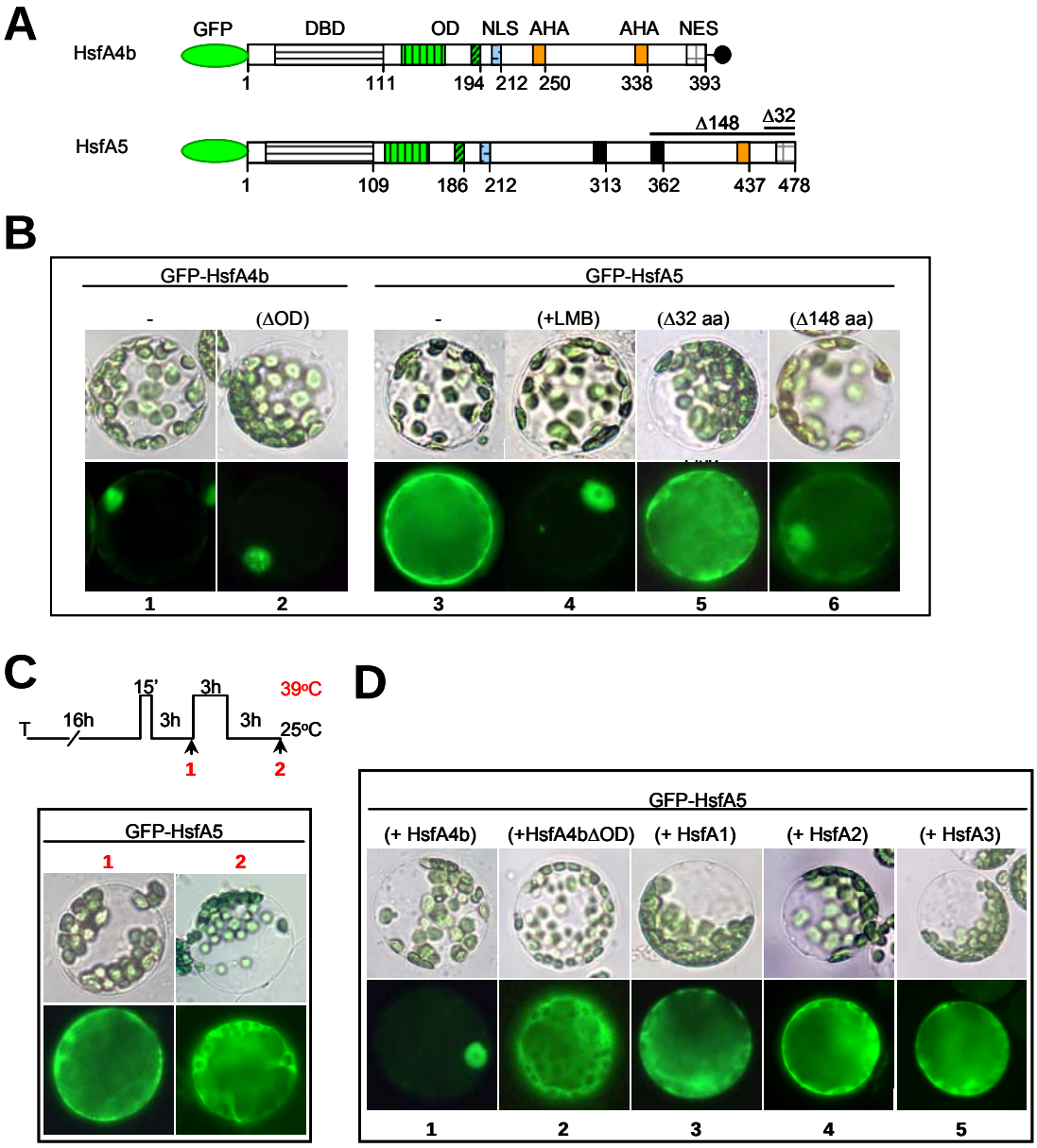


Figure 3.19 Intracellular localization of tomato HsfA4b and HsfA5.

(Figure modified from Baniwal *et al.* 2007)

A) Block diagrams to show GFP-Hsf fusion constructs used to transform tobacco protoplasts. For HsfA5, in addition to wt two c-terminally truncated forms were also tested.

B) Pictures of live tobacco protoplasts expressing indicated GFP-Hsf expression plasmids. Sample 1 corresponds to GFP-HsfA4b and samples 2 to its oligomerization domain deletion mutant (ΔOD). Samples 3 and 6 correspond to wt GFP-HsfA5 before and after treatment with LMB. Sample 5 and 6 correspond to the mutant forms of GFP-HsfA5 with 32 and 148 aa deleted from the c-terminus.

C) Effect of heat stress on intracellular localization of GFP-HsfA5. Samples of protoplasts expressing GFP-HsfA5 were heat stressed and examined for fluorescent signal at the indicated time points 1 and 2 (red). The heat stress regimen used is indicated in the *upper panel*, T stands for transformation.

virtually identical localization behavior as the wt HsfA5, whereas a large proportion of GFP-HsfA5Δ330 was retained in the nucleus (Fig. 3.19*B,* sample 5 and 6). These results imply that the region aa 330 - 440 contains a functional NES and the C-terminal VEHLTL is either non-functional or at least inefficient.

3.15.2 HsfA4b can affect intracellular distribution of HsfA5

Earlier it was found that HsfA2 needs o-expression with HsfA1a for its nuclear retention (Heerklotz *et al*. 2001). Based on the results about the interaction with HsfA4b, I investigated the possibility that the intracellular localization of HsfA5 might be influenced by HsfA4b. Indeed, co-expression of GFP-HsfA5 with HsfA4b caused strong nuclear localization of the former (Fig. 3.19*D*, sample 1). Evidently, the balance of nuclear import and export for the heterooligomers of Hsfs A4b and A5 is shifted towards the import reaction. No effect was observed by co-expression of GFP-HsfA5 with a deletion mutant of HsfA4b lacking its oligomerization domain or with Hsfs A1a, A2, A3 (Fig. 3.19*B*, samples 3 to 5 respectively). As control, similar to GFP-A4b, GFP-HsfA4bΔOD alone was also found to be localized in the nucleus (Fig. 3.19*B*, sample 2).

3.16 Visualization of HsfA4b and HsfA5 heterooligomers *in vivo*

A stringent and valuable method to demonstrate tight physical interaction between two proteins is the bimolecular fluorescence complementation (BiFC) analysis (Walter *et al*. 2004), because this approach enables visualization of the two interacting proteins in the normal cellular milieu. This test is based on the in vivo reconstitution two fragments of YFP by the interacting proteins fused to the N-terminal and C-terminal halves of the YFP. Plasmids encoding fusion proteins of tomato Hsfs A4b and A5 with the two complementary fragments (YN: aa 1-154; YC: aa 155-241) were transformed into the tobacco protoplasts (Fig. 3.20). Co-transformations of tobacco protoplasts with YN and YC fragments alone served as background control (Fig. 3.20*B*, sample 1). Protoplasts expressing HsfA4b-YN and HsfA4b-YC showed strong YFP complementation in the nucleus (Fig. 3.20*B*, sample 2). In contrast to this, samples expressing HsfA5-YN and HsfA5-YC gave extremely faint signals (Fig. 3.20*C*, sample 6). This could be the result of the general distribution of HsfA5 in the cytoplasm. Therefore, HsfA5-YN and HsfA5-YC were co-expressed together with HsfA4b and HsfA1a containing no fluorescent tag. Indeed, a clear nuclear YFP signal was detectable in the presence of HsfA4b (Fig. 3.20*C*, samples 7, 8, 9) but not of HsfA1a (sample 10). Interestingly, the nuclear detection of YFP fluorescence in sample 4 indicates the formation of (HsfA5-YN/HsfA5-YC)-HsfA4b heterotrimers or multimers of this type. As would be expected, co-expression of HsfA4b-YN and HsfA5-YC or *vice versa*, also resulted in strong nuclear YFP signals (Fig. 3.20*B*, samples 4). However, no complementation was observed when either one of the two Hsf was lacking

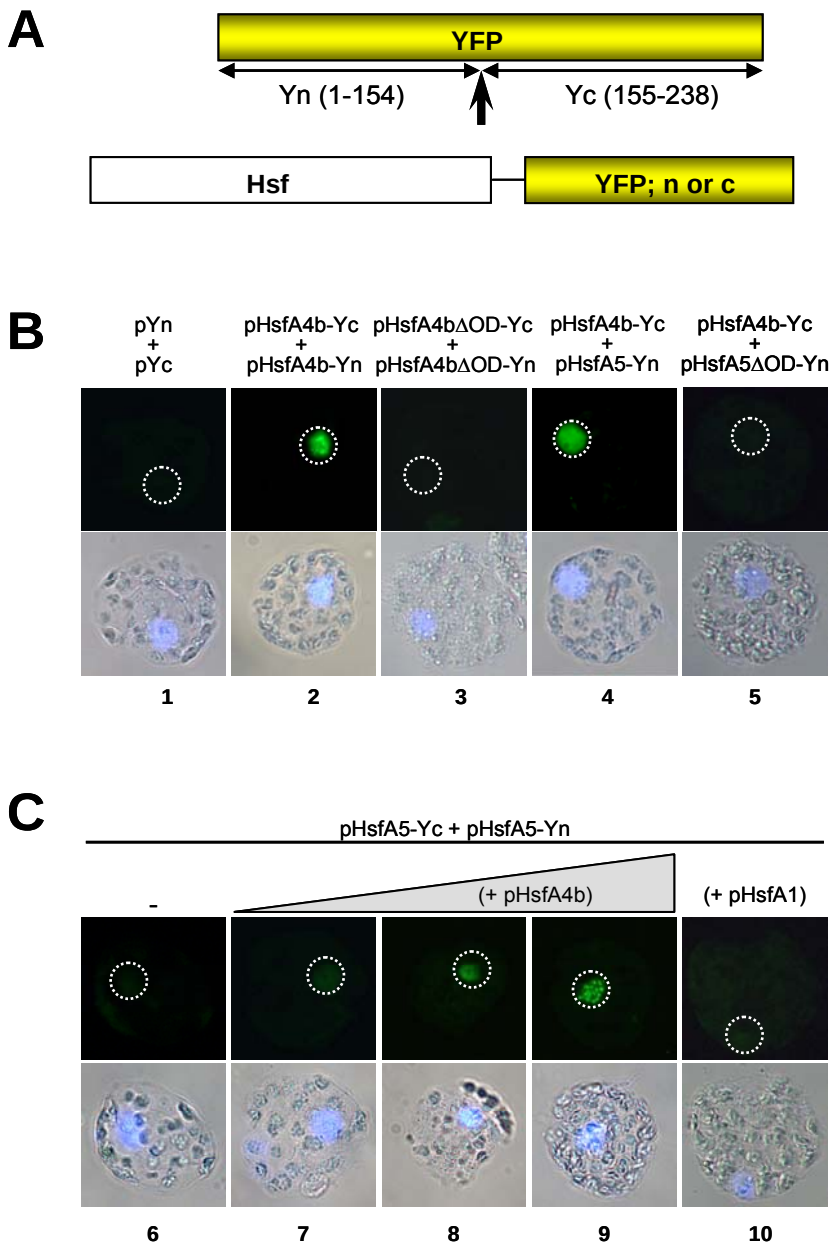


Figure 3.20 Visualization of HsfA4b-HsfA5 hetero-oligomers using Bimolecular fluorescence complementation (BiFC).

(Figure modified from Baniwal *et al.* 2007)

(A) Schematic representation of split YFP with complementary N- and C-terminal parts.

(B) Tobacco protoplasts (fixed) expressing split-YFP i.e. Yn/Yc and their fusions with w.t. or mutant forms of HsfA4b and HsfA5 as indicated on the top of each picture.

(C) Homo-oligomeric combination of HsfA5 was transformed (sample 6). HsfA4b-3HA and HsfA1 was co-expressed independently with the homo-oligomeric combination of HsfA5-Yn and HsfA5-Yc. Three different (increasing) amounts of HsfA4b-3HA were co-transformed (sample 7 to 9) whereas comparable highest amount of Hsf1 encoding plasmid was used (sample 10). In all samples the position of the nucleus was determined by DAPI staining of fixed cells and depicted as dotted circle in the pictures. A plasmid amount of 2.5 µg was used for each split YFP construct to transform $100x10^3$ tobacco mesophyll protoplasts.

its oligomerization domain (Fig. 3.20*B*, sample 3 and 5). These results with the BiFC technique clearly confirm the specificity and structural requirements of the interaction between Hsfs A4b and A5.

4. DISCUSSION

4.1 Hsf multiplicity in plants and cooperation between Hsfs of the tomato Hsf family

Compared to all other organisms with 1 to 3 Hsfs or Hsf-related transcription factors (Morimoto, 1998, Nakai, 1999, Pirkkala *et al*. 2001, Voellmy, 2004), the multiplicity of members of the Hsf family in plants is striking (Nover *et al*. 2001, Baniwal *et al*. 2004). Although our knowledge is still very limited, functional diversification seems to be the main reason for the coexistence of more than 20 Hsfs in plants. Remarkable cases of specialization by selective expression were reported for HsfA2 as strongly hs-induced protein (Scharf *et al*. 1990, Schramm *et al*. 2006) and for HsfA9 with exclusive expression during seed maturation (Kotak *et al*. 2007). On the other hand, the well studied examples of cooperation between three tomato Hsfs *i.e.* Hsfs A1, A2, and B1, impressively illustrated the extent of functional diversification in this family.

The oligomerization domain of Hsfs facilitates homo- and hetero-oligomerization that contributes considerably to this functional diversification. For instance, HsfA1a and HsfA2 are moderately strong activators in the homo-oligomeric state but they form heterooligomers that have super-activator properties. The remarkable effect resides mainly in the combination of different activator motifs (AHA motifs) essential for efficient recruitment of components of the transcription machinery (Chan *et al.* 2007).

Interestingly, the mode of synergistic interaction between HsfA1a and HsfB1 is completely different. They do not physically interact but they form a complex *via* interactions with CBP/p300 (CREB binding protein). The resulting ternary complex helps to assemble hs enhanceosome provided the promotor architecture allows binding of both Hsfs in close vicinity (Bharti *et al*. 2004). In addition, HsfB1 can also

function with other activator proteins to restore transcription of house keeping genes. In summary, HsfA1a, HsfA2, and HsfB1 together form a functional triad for the essential three phases of the hs response, *i.e.* the triggering (HsfA1a as master regulator), maintenance and high efficiency of hs gene transcription (cooperation of HsfA1a/A2/A3 heterooligomers with HsfB1) as well as for the restoration of house-keeping gene transcription during the recovery phase (HsfB1 with yet unknown house-keeping transcription factors).

4.2 HsfA4/A5 subgroup reveals novel aspects of Hsf cooperation

Compared to earlier described examples of Hsfs A1a, A2, A3, A9, and B1 (see Introduction section) the situation with Hsfs A4 and A5 described in this thesis opens a completely novel aspect of Hsf cooperation. Both, HsfA4 and HsfA5 homooligomerize and bind to corresponding HSE motifs. But both Hsfs have strong tendency for heterooligomerization. The stoichiometry of these heterooligomers is not yet completely clear but based on observations using split YFP technique; it certainly contains two HsfA5 and at-least one HsfA4. Although the HsfA4/A5 complex localizes to the nucleus, it is transcriptionally inactive due to the impairment of DNA binding. However, in the present studies, it could not be clarified in further details.

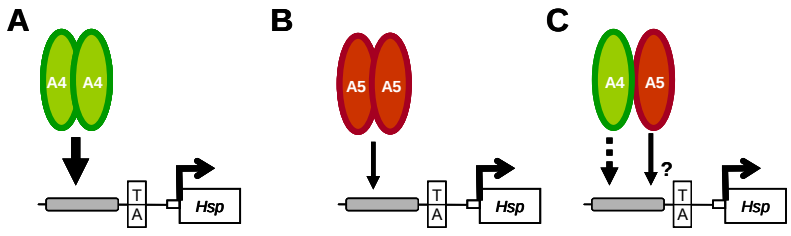


Figure 4.1 Schematic diagram representing the interplay of HsfA4 and HsfA5 on the promoters of hs genes. HsfA4 and HsfA5 homooligomerize and bind to corresponding HSE motifs (A, B). Both Hsfs have strong tendency for heterooligomerization and the HsfA4/A5 heterooligomers localize in the nucleus. These complexes are transcriptionally inactive due to the impairment of DNA binding which is essential for the activator function (C).

4.3 The activator/repressor relationship of HsfA4/A5 is likely to be a general feature of plant Hsf families

The inhibition of HsfA4 activity by HsfA5 is found for both tomato and Arabidopsis (Figs. 3.11 and 3.17 respectively) as well as for corresponding cross-species combinations of these Hsfs. Considering the conservation of Hsfs A4 and A5 (Table 4.1), it is tempting to speculate that it is indeed a fundamental feature of the Hsf system in plants. The inhibitory function of HsfA5 requires only its OD. Obviously, no additional factors, *e.g.* a putative co-repressor recruited by the C-terminal domain, are involved. In view of these results, it is also not reasonable to discuss a simple shielding mechanism for the AHA motifs of HsfA4 by a putative internal repressor domain of HsfA5. Evidently, the repressor effect mainly results from the interference with the oligomeric state of HsfA4b, which is essential for efficient DNA binding and activator functions. Consistent with this interpretation, HsfA1a, which itself is insensitive to the inhibitory effect of HsfA5, was made sensitive by exchanging its oligomerization domain with that of HsfA4b. Thus, provided the compatible oligomerization domains, the repressor effect can be imparted onto other Hsfs. The whole molecular context of HsfA4 is not required.

The stringent interaction behaviour of Hsfs A4 and A5 demonstrates an unexpected specificity generated by their oligomerization domains. Unfortunately, sequence inspection of the HR-A/B regions of HsfA1a/A2-type on the one hand and HsfsA4/A5-type on the other gave no immediate clue to the basis of this specificity. Moreover, pull-down assays and yeast two-hybrid interaction tests (Fig. 3.18) clearly indicate that the formation of heterooligomers is preferred. Very likely, this tendency is crucial for the strong repressor effect of HsfA5 on HsfA4 activity. In all cases investigated, deletion or heterologous replacement of the oligomerization domain in one of the two partner Hsfs abolished the repressor effect.

4.4 Biological implications of HsfA4/A5 interaction

Are specific genes addressed by HsfA4? All our assays in this work were based on Hsf-dependent reporters, which respond equally well to HsfA1a/A2 and to HsfA4. Although a considerable collection of different reporters were tested, I never found any striking differences (Table 3.2). However, this can not exclude a situation in plants, where combination of HsfA4 with other tissue-specific transcription factors creates promoter specificity not detectable in our reporter assays. This argument also holds true for the function of HsfA4 for hs-induced gene expression. The dominant role of HsfA1a as master regulator (Fig. 1.4) may not be valid to the same extent for all tissues and developmental stages.

An essential aspect of the discussion about the specialized function of Hsfs A4 and A5 in plants is their expression profile. From EST and whole genome sequence data bases of rice and Arabidopsis I identified, a single HsfA5 but usually one or two A4 Hsfs. All members of the HsfA5 subgroup are much more conserved among each other than the members of the HsfA4 subgroup (Fig. 3.3 and the sequence details compiled in Table 4.1). The detection of numerous ESTs indicates that representatives of the A4/A5 group are well expressed in different plant tissues (Fig. 3.4 and S. Baniwal, Diploma thesis 2002). Analysis of the Arabidopsis microarray data bases confirmed that Hsfs A4a, A4c and A5 mRNAs are indeed found in many tissues, albeit at very different levels which change with the developmental stages and stress conditions (Fig. 3.5). Usually, the levels of HsfA5 mRNA in Arabidopsis are markedly lower than those of Hsfs A4a and A4c. This contrasts to the high abundance of HsfA5-specific ESTs in the data bases of other plants. These considerations are helpful, but it should be recalled that all data are based on RNA analyses which may not give direct information about the corresponding protein levels.

A: HsfA4	NLS		AHA		NES
1.LeA4b	ASRKRRLL	113aa	NDVFWQQFLTETPGCTEPQQVENKGINE	12aa	YWWNRGVNLENLAERMGHLSSPATGS*
2.StA4b	KRRLLV	136aa	NDVFWQQFLTETPGCTEPQEVENKGINE	12aa	YWWNSGVNVENLAERMGHLSSPATG
3.AtA4c	HERRKRRF	83aa	NDDFWEQCLTENPGSTE-QQEVQSERRD	13aa	YWWNSGNVNNIT---------EKAS*
4.AtA4a	NERKRRFP	127aa	NDGFWQQFFSENPGSTE-QREVQLERKD	11aa	CWWNSRNVNAITEQ-LGHLTSSERS*
5.LeA4a	NERKRRLP	130aa	NDVFWEQFLTENPGSTDVKPEREDMESK	11aa	FWWNRKTVISLTEQ-LGHLTPAE*
6.NtA4a	NDRKRRLP	132aa	NDIFWEQFLTENPGSVD-ASEVQSERKD	15aa	FWWNMKSVNSLAEQ-LGHLTPAEKT*
7.HaA4a	NSRKRRLL	113aa	NDVFWEQFLTETPGSGD-TQEVQSERRD---VTKPLWGTHNLGKITEK-MGNLGPGKLDVR*		
8.CsA4a	HDRKRRLP	128aa	NDVFWEQFLTENPGSSD-AQEVQSERKE	15aa	FWWNMRNVNSLAEQ-MGHLTPAERT*
9.InA4a	PDRKRRLP	128aa	NDLFWEQFLTENPGSTDAPTDVLSERKN	15aa	FWWSVKSVNNLAEQ-LGHLTPAERT*
10.GmA4a	MDRKRRLP	128aa	NDIFWERFLTENPGSSEMQEAQSEREDS	7aa	FWWNIRNVNNPPEQ-MGHLSKAEQT
11.GmA4c	LDRKRRLP	128aa	NDVFWEQFLTEDPGASE-TREVQSERKD	15aa	FWWNKRNANNLPEQ-MGHVGQAEKT*
12.MtA4a	MERKRRLP	128aa	NDVFWEQFLTEDPGASE-AQEVQSERKD	15aa	FWWNMRKSNNHPEQ-MGHVSQVEKI*
13.ZmA4a	HGKKRRLP	163aa	NDGFWQQFLTEQPGPD-VHQEAQSERRD	16aa	FWWGKKNVEQMREK-LGRLTSVEKT*
14.OsA4a	HRKKRRLP	167aa	NDGFWQQFLTEQPGSSDAHQEAQSERRD	15aa	LWWGKRNVEQITEK-LGLLTSTEKT*
15.SoA4a	HGKKRRLQ	162aa	NDGFWQQFLTEQPGS-DAHHEAQSERRD	15aa	FWWGKKNVEQMTEK-LGHLTSVEKT*
16.AcA4d	SSKKRRVP	161aa	NDLFWERFLTETP	9aa	HDADCKRETPEPKDHVRIGIDRNWFNRRGNVEQIIEQMEHL*
17.OsA4d	FSKKRRVP	180aa	NDVFWERFLTETP	11aa	SPKDDVKAELGCNGFHHREKVDQITEQMGHLASAEQTLHT*
18.ZmA4d	truncat.>136aa		NDVFWERFLTDAA	7aa	EAKEDVKTAVDRCCPRL-QDNVDQITEQMGQLDSA---SYAPENY*
19.SoA4d	truncat.>159aa		NDVFWERFLTD--	5aa	EAKEDVKAAVNRSCLRL-QDNGDQITEQMGQLDSAENDSYAPQNY*

B: HsfA5	NLS		SNED		CLLLAS		AHA		(NES)
1.LeA5	FSKKRRLP	52aa	STQSSNED	78aa	CQLNLSLAS	63aa	NDVFWEQFLTERPGCSDNEEASS	23aa	RKVEHLTL*
2.StA5	FSKKRRLL	52aa	STQSSNED	78aa	CQLNLSLAS	63aa	NDVFWEQFLTERPGCSDNEEASS	23aa	RKVEHLTL*
3.AtA5	YNKKRRLP	53aa	SIQSSNEE	68aa	CHLNLTLAS	55aa	NDVFWEQFLTERPGSSDNEEASS	23aa	KNIEQLTL*
4.AfA5	INKKRRLP	50aa	SAQSSDED	67aa	CHLNLTLAS	73aa	NDGFWEQYLTERPGSPDTEEASS	24aa	SDMEQLTL*
5.NbA5	FSKKRRLP	52aa	STQSSNED<70aa		xxLNLSLAS	63aa	NDVFWEQFLTERPGCSDNEEASS	23aa	RKVEHLTL*
6.GmA5	AYKKRRLP	52aa	STQSSNED	72aa	CQLNLTLAS	74aa	NDVFWEQFLTERPGCSDNEEAIS	23aa	KNMDQLTL*
7.MtA5	YNKKRRLP	52aa	STQGSNED	72aa	CQLNLTLAS	74aa	NDVFWENFLTERPGCSDNEEAIS	23aa	KNMDNLTL*
8.ZmA5	truncat.		STQSSHED	54aa	CHLNLSLAS	84aa	NDKFWEQFLTERPGCPEAEEASF	20aa	RDMGQLKL*
9.OsjA5	FNKKRRLP	54aa	STQSSNED	54aa	CHLSLTLAS	75aa	NDKFWEQFLTERPGCSETEEASS	24aa	EDVEQLKL*
10.SbA5	FHKKRRLP	54aa	STQSSHED	54aa	CHLNLSLAS	83aa	NDKFWEQFLTERPGCSEAEEASS	20aa	RDMGQLKL*
11.SoA5	truncat.>50aa		STQSSHED	54aa	CHLNLSLAS	82aa	NDKFWEQFLTERPGCSEAEEASS	19aa	RDMGQLKL*

Table 4.1 Sequence alignment of C-terminal signature sequences of HsfA4 and HsfA5.

Amino acid sequences were derived from the EST databases Abbreviations (accession numbers): **At** *Arabidopsis thaliana* HsfA4a (At4g18880), HsfA4c (At5g45710), HsfA5 (At4g13980); **Bv**, *Beta vulgaris* HsfA5 (BQ488901); **Cs**, *Citrus sinensis* HsfA4a (DY270414), HsfA5 (DY300016); **Gh**, *Gossypium hirsutum* HsfA5 (DT548305); **Gm**, *Glyine max* HsfA4a (TC135284), HsfA5 (BM270993); **Ha**, *Helianthus annuus* HsfA5 (DY932755); **Hv**, *Hordeum vulgare* HsfA4a (TC42448), HsfA5 (TC151027+BQ759144); **Le**, *Lycopersicon esculentum* HsfA4a (BT014619), HsfA4b ((TC107140), HsfA5 (TC155271); **Lj**, *Lotus japonicus* HsfA4a (AP004978); **Ls**, *Lactuca sativa* HsfA4a (DY973605), HsfA5 (DY974369); **Ms**, *Medicago sativa* HsfA4a (AF494082); **Mt**, *Medicago truncatula* HsfA4a (TC79769), HsfA5 (TC79192); **Nb**, *Nicotiana benthamiana* HsfA5 (CK287755); **Nt**, N. *tabaccum* HsfA4a (AB014484); **Os**, *Oryza sativa japonica* HsfA4a (AP004879), HsfA4d (AC111015), HsfA5 (AP004999); **Pa**, *Phaseolus aureus* HsfA4a (AY052627); **Sb**, *Sorghum bicolor* HsfA4a (BM322601); **So**, Saccharum officinarum HsfA5 (CA264007); **St**, Solanum tuberosum HsfA4b (BG591987), HsfA5 (TC112724); **Ta**, *Triticum aestivum* HsfA4d (CV766704), HsfA5 (CJ655373); **Zm** *Zea mays* HsfA4a (X92943), HsfA5 (EE174627) **Ac**, *Agrostis capillaris*; **Af**, *Aquilegia formosa*; **In**, *Ipomea nil*.

4.5 Hsp deregulation mediated apoptosis and role of HsfA4/A5 subgroup

Intriguing hints for a specialized function of A4-type Hsfs came from the analyses of rice HsfA4d mutant showing spontaneous necrotic lesions in mature leaves due to evident hypersensitivity to mild stress challenges (Yamanouchi *et al*. 2002). On the other hand, transgenic Arabidopsis plants over-expressing a dominant negative form of HsfA4a had defects in their response to oxidative stress (Davletova *et al*. 2005). It is tempting to speculate that we are actually dealing with the same HsfA4-dependent syndrome in rice and Arabidopsis, because in both cases reactive oxygen species play key roles as stressors and signals.

The repressor function of HsfA5 reminds of an earlier report from mammals and nematodes about an Hsf-binding protein (HSBP1), which by virtue of an extended hydrophobic heptad repeat region, interacts selectively with Hsf1 and attenuates the hs response (Satyal *et al*. 1998). HSBP encoding genes and corresponding ESTs were also identified in plants (Fu *et al*. 2002). An interesting aspect emerged, when a maize mutant *emp2* (empty pericarp 2) was identified as a defect in one of the two HSBPs. In the *emp2* mutant, endosperm and embryo development are deeply disturbed and this coincides with an up-regulation of Hsp encoding genes (Fu *et al*. 2002), suggesting EMP2 to be essential for maintaining chaperone homeostasis during embryo development. Based on yeast two hybrid interaction tests, the two maize HSBPs were shown to interact selectively with very few class A Hsfs including HsfA4a for HSBP2 and

Hsfs A4d and A5 for EMP2. Moreover, mutations in few conserved hydrophobic residues in the HsfA4a OD abolished the interaction with HSBP2 (Fu *et al*. 2006).

In summary, the most plausible mechanism for the repressor role of HsfA5 is based on its high affinity to interact with HsfA4 to generate inactive heterooligomers. It remains to be examined whether the balance between active HsfA4 homotrimers and inactive HsfA4/HsfA5 heterotrimers could be influenced by modifications, *e.g.* as a result of oxidative stress as nicely summarized by Miller and Mittler, or whether the shuttling of HsfA5 between nucleus and cytoplasm plays a role in such a regulatory mechanism. Similar to the highly selective function of HsfA9 as part of the ABA-controlled program of seed development, the special role of Hsfs A4 and A5 may be restricted to certain developmental stages and/or to biotic or abiotic stress challenges. I hypothesise that, similar to HSBPs, HsfA5 may represent a novel type of selective repressor, regulating the function of A4-type Hsfs in higher plants.

4.6 The discrepancy between the functional modules and activator function of HsfA5

When tested in reporter assays with Hsf-dependent promoter constructs, tomato HsfA4b was functionally equivalent or even stronger than Hsfs A1 and A2, whereas HsfA5 was completely inactive (Fig. 3.6). This result was surprising, since HsfA5 has all necessary functional elements of a bona fide activator Hsf. It has a functional DNA binding domain (Fig. 3.8) and harbours a typical and highly conserved AHA motif in its C-terminal part shown earlier to be crucial for the

activator function of Hsfs (Treuter *et al*. 1993, Bharti *et al*. 2000, Döring *et al.* 2000, Kotak *et al*. 2004, Czarnecka *et al*. 2004).

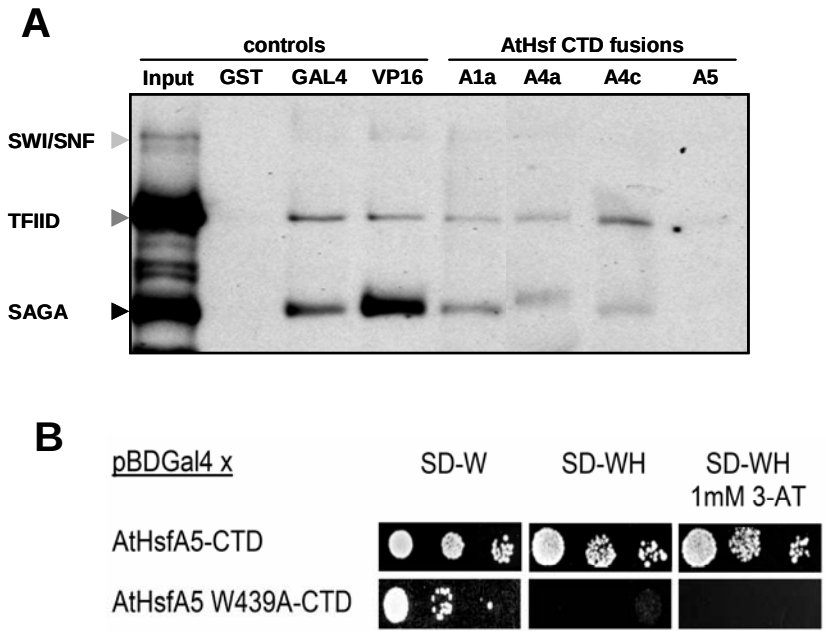


Figure 4.2 GST pull-down and yeast mono hybrid assays using Arabidopsis Hsfs.
A) GST-AtHsfCTD as baits to pull down transcriptional complexes containing the indicated components.
B) Growth of yeast cells containing Gal4BD-AtHsfA5 as bait to examine the activator potential.
(Figure modified from Kotak *et al.* 2004 and of M. Port, Ph.D. thesis 2006)

The AHA motifs represent the contact sites to interact with various complexes of the transcriptional machinery, *e.g.* SWI/SNF, TFIID, and SAGA (Kotak *et al.* 2004). Using Pull-down technique, interactions were readily detectable for Arabidopsis Hsfs A4a and A4c, but not for HsfA5 (Fig. 4.2*A*). Surprisingly, fusion of the C-terminal domain of HsfA5 to the Gal4 DBD gave a weakly active activator protein in yeast, and mutation of the Trp residue in the predicted AHA motif (-F**W**EQFL- → -F**A**EQFL-) abolished this activity (Fig. 4.2*B*). These results indicate that the predicted AHA motif is functional but not in its natural context of the wt HsfA5 (Kotak *et al*. 2004).

4.7 Repression of HsfA4 may not be the exclusive function of HsfA5

Although the present work focussed on different aspects of HsfA4 and HsfA5 interaction, additional roles of HsfA5 can not be ruled out. Since HsfA5 stimulated the transcriptional activity of other class A Hsfs, Hsfs A1a, A2, A3 (Fig. 3.12) it may be speculated that HsfA5 serves in the fine tuning of the other aspects of hs gene expression as well. In addition to this HsfA5 might cooperate with yet unidentified transcription factors as shown for HsfB1 (see Introduction). Interestingly using yeast two hybrid system Markus Port identified several potential interacting partners of HsfA5 (M. Port, Ph.D. thesis 2006). Detailed investigations on these proteins are lacking.

As well documented for HsHsf1 (see Introduction) various post-translational modifications may modulate its functional properties. It will be interesting to examine if HsfA5 or HsfA4 undergo tissue or stress specific modifications which in turn would determine their individual and/or network based behaviours. It might turn out that HsfA5 recognizes a exclusive set of genes in response to stress or developmental cues. Finally, it should be noted that use of transgenic plants carrying knock-down and overexpression of these Hsfs either alone or in different combinations will be essential to understand the function of HsfA4 and HsfA5 as part of the complex plant stress response system.

5.1. Summary

Compared to all other organisms with 1 to 3 heat stress transcription factors (Hsfs) or Hsf-related factors, plants have extraordinarily large Hsf families with more than 20 Hsfs. Plant Hsfs are classified into three classes according to their oligomerization domains which is built of hydrophobic heptad repeats (HR) in two parts, HR-A and HR-B. Both parts may be immediately adjacent (class B), or they are separated by insertion of 21 (class A) and 7 amino acid residues (class C).

In plant Hsf family, detailed investigations are so far limited to Hsfs A1a, A2, A3, A4d, A9, and B1. They strongly indicate functional diversification to be the main reason for the coexistence of multiple Hsfs. As an example the functional triad of HsfA1a, HsfA2, and HsfB1 is essential for all three phases of the hs response, (i) the triggering of the response by HsfA1a as master regulator, (ii) the maintenance and high efficiency of hs gene transcription by cooperation of HsfA1a with Hsfs A2 and B1, and finally, (iii) the restoration of house-keeping gene transcription during the recovery phase mediated by HsfB1 in cooperation with house-keeping transcription factors.

The results presented in this thesis for Hsfs A4 and A5 open completely different aspects of functional diversification and cooperation of Hsfs. HsfA4 and HsfA5 homooligomerize and bind to corresponding HSE motifs. But in contrast to the highly active HsfA4, HsfA5 is completely inactive as transcriptional activator. Yeast two hybrid and GST pull-down techniques showed that both Hsfs have strong tendency for heterooligomerization. Using fluorescence microscopy the

HsfA4/A5 heterooligomers were found to localize in the nucleus. These complexes are transcriptionally inactive due to the impairment of DNA binding. The repressor function of HsfA5 requires only its OD and no additional factors, *e.g.* a putative co-repressor recruited by the C-terminal domain, are involved. Evidently, the repressor effect mainly results from the interference with the oligomeric state of HsfA4b, which is essential for efficient DNA binding and activator functions.

EST database search revealed that plants have a single HsfA5 and usually two A4-type Hsfs. Using bioinformatics tools, Hsfs A4 and A5 were found to be phylogenetically closely related and clearly distinct from the other members of the Hsf family. On the basis of RT-PCR and Microarray data the representatives of the A4/A5 group are well expressed in different plant tissues albeit at very different levels which change with the developmental stages and stress conditions

In rice and Arabidopsis, HsfA4 functions as an anti-apoptotic factor for stress induced oxidative damages. Based on my results, I hypothesize that HsfA5 functions as a novel type of selective repressor, regulating the function of A4-type Hsfs in plants. Considering the high sequence conservation with in plant Hsf family, it is tempting to speculate that this role of Hsf4/A5 pair is a fundamental feature of the Hsf system in plants.

5.2 Zusammenfassung in deutscher Sprache:

- Im Vergleich zu anderen eukaryotischen Organismen haben Pflanzen ein ungewöhnlich komplexes System der Streßantwort, das auch eine Familie mit mehr als 20 Regulatorproteinen, sog. Hitzestresstranskriptionsfaktoren (Hsfs), einschliesst. Umfassende Untersuchungen zur funktionellen Diversifizierung der Hsfs sind bisher allerdings auf wenige Vertreter beschränkt (Hsfs A1, A2, A3, A9 und B1). Als Beispiel kann die funktionelle Triade von HsfA1, A2 und B1 in Tomate angeführt werden, die in typischer Weise die drei essentiellen Phasen einer Hitzestressantwort begleiten: (i) Auslösung der Antwort durch den Masterregulator HsfA1 einschliesslich der Neubildung von HsfA2 und HsfB1, (ii) Verstärkung der Genxepression bei einer Langzeitstressbelastung durch Kooperation von HsfA1 mit HsfA2 und B1 und schliesslich (iii) Wiederaufnahme der Haushaltsgenexpression in der Erholungsphase unter Mitwirkung von HsfB1.
- Die folgenden Ergebnisse über die Rolle von Hsfs A4 und A5 eröffnen eine ganz neue Sicht auf die Funktionsvielfalt von Hsfs. Beide Hsfs binden an die entsprechenden Promoterelemente (heat stress element, HSE); aber im Gegensatz zu der starken Aktivität von HsfA4 im Reporterassay in Tabakprotoplasten sind alle Vertreter der HsfA5 Gruppe inaktiv. Bei Coexpressionsexperimenten konnte gezeigt werden, dass die Anwesenheit von HsfA5 die Aktivität von HsfA4 behindert. Nach allen Kriterien muss HsfA5 als ein spezifischer Repressor von HsfA4 bezeichnet werden. Durch in vitro Pull-down Experimente und Tests im Hefe-Zweihybridsystem konnte gezeigt werden, dass HsfA4 und HsfA5 eine starke Tendenz zur Bildung von Heterooligomeren haben, und diese werden in Tabakprotoplasten im Zellkern gefunden.
- Aus Datenbankuntersuchungen geht hervor, dass alle Pflanzen im allgemeinen zwei HsfA4 und einen HsfA5 haben. Bei Sequenzvergleichen finden sich die

Mitglieder der HsfA4/A5 Gruppe klar getrennt von anderen Vertretern der Hsf Familie. Die Expression der Hsf A4 und A5 mRNA in verschiedenen Geweben von Tomate wurde durch RT-PCR und für Arabidospsis aus Daten international zugänglicher Mikroarrayanalysen ermittelt. Alle Vertreter sind im allgemeinen gut exprimiert, wenn auch der mRNA Spiegel in Abhängigkeit von Gewebe oder von Stressbehandlungen Schwankungen unterworfen ist.

- Die Bedeutung der Ergebnisse von HsfA4 als starkem Aktivator und HsfA5 als spezifischem Repressor der HsfA4 Funktion wird dadurch unterstrichen, dass durch Untersuchungen in Reis und Arabidopsis HsfA4 als antiapoptotischer Faktor in Verbindung mit oxidativem Stress gekennzeichnet wurde. Die starke Konservierung innerhalb der HsfA4/A5 Gruppe macht wahrscheinlich, dass es sich um ein allgemeines Phänom bei Pflanzen handeln könnte. Allerdings müssen die biologischen Details unter Einbeziehung transgener Pflanzen mit knock-out bzw. Überexpression dieser Hsfs noch geklärt werden.

5.3 Ausführliche Zusammenfassung in deutscher Sprache:

- Im Vergleich zu anderen eukaryotischen Organismen haben Pflanzen ein ungewöhnlich komplexes System der Streßantwort, das auch eine Familie mit mehr als 20 Regulatorproteinen, sog. Hitzestresstranskriptionsfaktoren (Hsfs), einschliesst. Umfassendere Untersuchungen zur funktionellen Diversifizierung der Hsfs sind bisher allerdings auf wenige Vertreter beschränkt (Hsfs A1, A2, A3, A9 und B1). Als Beispiel kann die funktionelle Triade von HsfA1, A2 und B1 in Tomate angeführt werden, die in typischer Weise die drei essentiellen Phasen einer Hitzestressantwort begleiten: (i) Auslösung der Antwort durch den Masterregulator HsfA1 einschliesslich der Neubildung von HsfA2 und HsfB1, (ii) Verstärkung der Genxepression bei einer Langzeitstressbelastung durch Kooperation von HsfA1 mit HsfA2 und B1 und schliesslich (iii) Wiederaufnahme der Haushaltsgenexpression in der Erholungsphase unter Mitwirkung von HsfB1. Die folgenden Ergebnisse über die Rolle von Hsfs A4 und A5 eröffnen eine ganz neue Sicht auf die Funktionsvielfalt von Hsfs.
- Umfangreiche bioinformatische Untersuchungen in internationalen Datenbanken ergaben mehr als 18 Hitzestreßtranskriptionsfaktoren (Hsfs) in Tomate, die in ihrer Struktur mit den 21 Hsfs von *Arabidopsis thaliana* und den 23 Hsfs von Reis (*Oryza sativa*) verglichen wurden. Besonders auffallend ist eine klar abgetrennte Gruppe von nahe verwandten Hsfs, die in zwei Untergruppen zerfällt. Dieses sind Hsfs A4 und A5, die bei allen Pflanzen offensichtlich weit verbreitet sind, wie die Analysen der EST (expressed sequence tags) Datenbanken ergeben. Stets findet man 1-3 Vertreter von HsfA4 und einen einzigen Vertreter von HsfA5.
- Neben den bei allen Hsfs konservierten Modulen in der N-terminalen Hälfte (DNA Bindungsdomäne, Oligomerisierungsdomäne (HR-A/B-Region) und der benachbarten Kernlokalisationssequenz (NLS)) haben wir eine Reihe typischer

Erkennungssequenzen in den C-terminalen Teilen dieser Hsfs identifiziert. Diese sog. Signatursequenzmotive erlauben es, viele der bisher nicht identifizierten Hsf Fragmente aus den EST-Datenbanken eindeutig zuzuordnen. Der C-Terminus der HsfA4/A5 Gruppe schliesst auch ein sog. AHA-Motif ein, das nach Untersuchungen an anderen Hsfs für die Wechselwirkung mit Komplexen der Transkriptionsmaschine und damit für das Aktivatorpotential verantwortlich ist.

- Untersuchungen zur Expression der Vertreter dieser Gruppe bauen ausschliesslich auf der Analyse der mRNA auf, da bisher ein Nachweis der Proteine in nativen Geweben nicht gelungen ist. Gesamt RNA Präparationen aus verschiedenen Geweben der Tomate wurden mit RT-PCR analysiert, während für Arabidopsis die entsprechenden Daten aus Mikroarrayanalysen in internationalen Datenbanken entnommen wurden. Im allgemeinen zeigen die beiden Hsfs A4 eine starke Anreicherung der Expression in bestimmten Geweben bzw. nach Stressbehandlung, während HsfA5 eher konstitutiv in allen Geweben gefunden wird. Diese Daten geben wertvolle Hinweise für weitere Untersuchungen, können aber die fehlende Proteinanalyse naturgemäss nicht ersetzen.
- Die Funktion von HsfA4 und HsfA5 als HSE-abhängige Transkriptionsaktivatoren wurde in Tabakmesophyllprotoplasten mit Hilfe entsprechender Reportergene untersucht. Dabei wurden auf der einen Seite Plasmide mit verschiedene GUS (ß-Glucuronidase) Reporterkassetten und auf der anderen Seite die endogenen Hitzestressgene des Tabaks genutzt. Während im ersten Fall der Nachweis mit Methylumbelliferon-glucuronid als chromogenem Substrat erfolgt, müssen im zweiten Fall Westernblots mit Antikörpern gegen eines der prominenten Hsps (Hsp17-CI) angefertigt werden. Der grössere Aufwand ist gerechtfertigt, weil die Analyse der Hsf Wirkung auf die Hsp-codierenden Gene im Chromatin ausschliessen, dass die Ergebnisse durch Artefakte mit den Plasmid-codierten

Reportergenen verfälscht werden. Erstaunlicherweise waren die Ergebnisse mit beiden Typen von Reportern sehr ähnlich.

- Wie andere Hsfs (Hsfs A1, A2 und A3) sind auch die Hsfs der A4-Untergruppe vergleichsweise gute Aktivatoren. Tatsächlich handelt es sich bei dem hier am häufigsten genutzten Tomaten HsfA4b um den besten Aktivator, den wir bisher kennen. Dagegen waren alle HsfA5 vollständig inaktiv.
- Auf der Suche nach der Grundlage für diese Unterschiede zwischen Hsf A4 und HsfA5 haben wir die Fähigkeit zur DNA-Bindung in einem speziellen Assay (Repressorassay) verglichen. Beide Typen von Hsfs haben vergleichbare DNA-Bindungsaffinitäten. Auch erfüllte der N-terminale Teil des HsfA5 in Fusion mit dem C-Terminus von HsfA2 oder anderen Aktivatordomänen alle Voraussetzungen für einen funktionfähigen Hsf, d.h. offensichtlich beruht die Inaktivität von HsfA5 auf speziellen Gegebenheiten im Kontext des Gesamtproteins.
- Neue Einblicke in die mögliche biologische Funktion von HsfA5 wurden durch Coexpressionsexperimente mit HsfA4 bzw. anderen Hsfs erhalten. Das Aktivatorpotential von HsfA4 kann in Gegenwart steigender Mengen von HsfA5 vollständig blockiert werden. Diese Funktion von HsfA5 als Repressor von HsfA4 gilt sowohl für die beiden getesteten Vertreter aus Tomate als auch für die Vertreter aus Arabidopsis. Interessanterweise werden andere Hsfs (Hsfs A1, A2 und A3) in ihrer Funktion von der Anwesenheit von HsfA5 nicht gestört. Das gilt ebenso für die endogene Hitzestressantwort in den Tabakprotoplasten, die von HsfA1 als Masterregulator (s. o.) aber nicht von HsfA4 abhängig ist.
- Die Analyse der funktionellen Anatomie von HsfA5 konnte durch Coexpression von Fragmenten des Transkriptionsfaktors mit HsfA4 ermittelt werden. Für die Repressorwirkung wird ausschliesslich die Oligomerisierungsdomäne (OD) von HsfA5 benötigt, d.h. alle HsfA5-Derivate ohne funktionsfähige OD sind auch keine

Repressoren. Offensichtlich beruht der Mechanismus darauf, dass die für die Aktivatorwirkung notwendige Oligomerisierung von HsfA4 in Anwesenheit von HsfA5 gestört ist. Diese Schlussfolgerung wird durch Analysen eines Hybrid-HsfA1 gestärkt, in dem die eigene OD durch die des HsfA4 ersetzt wurde. Diese veränderte Form von HsfA1 ist empfindlich für die Anwesenheit von HsfA5.

- Die Selektivität der Wechselwirkung zwischen HsfA4 und HsfA5 wurde durch vier Versuchsansätze verifiziert:
  1. In vitro Pull-down Experimente mit rekombinanten Proteinen aus *E. coli* belegten die preferentielle Bildung von HsfA4/A5 Heterooligomeren.
  2. Diese Ergebnisse wurden durch Proteininteraktionstests in vivo mit dem Hefe-Zweihybridsystem bestätigt.
  3. In Tabakprotoplasten wurde die intrazelluläre Lokalisation von HsfA4 und HsfA5 durch Expression entsprechender Fusionsproteine mit GFP (green fluorescent protein) untersucht. HsfA4 befindet sich nahezu ausschliesslich im Zellkern, während HsfA5 typische Eigenschaften eines Shuttleproteins zwischen Kern und Cytoplasma aufweist. Auf Grund des dominanten Kernexports wird es zum überwiegenden Teil im Cytoplasma gefunden. Wenn man jedoch den Export durch das Antibiotikum Leptomycin B blockiert bleibt HsfA5 im Zellkern. Der gleiche Effekt kann allerdings durch Coexpression mit HsfA4 erreicht werden, weil offensichtlich in dem Heterooligomeren HsfA4/A5 die starke NES des HsfA5 unzugänglich ist.
  4. Unter Verwendung des sog. Split-YFP- oder BiFC-Systems konnte die direkte und selektive Wechselwirkung zwischen den beiden Partner Hsfs eindrucksvoll bestätigt werden. Die Komplementierung der Fluroreszenz des YFP (yellow fluorescence protein) aus den beiden Hälften funktioniert nur, wenn die beiden

interagierenden Hsf Partner über eine intakte und kompatible OD verfügen. Eine Komplementierung mit HsfA1 als Partner findet nicht statt.

- Die Bedeutung der Befunde beruht darauf, dass zum ersten Mal ein Hsf als ein selektiver Repressor identifiziert wurde und dass der aktive Partner (HsfA4) wichtige Funktionen für die basale Stresstoleranz der Pflanzen haben könnte. Darauf deuten Befunde an einer Reismutante mit einem Defekt in einem der beiden HsfA4 hin. Bei dieser Mutante treten auch unter normalen Kulturbedingungen spontane Nekrosen auf den Blättern auf, z. B. ausgelöst durch die mechanischen Reize beim Giessen. Diese Überempfindlichkeit wäre natürlich unter natürlichen Umständen verhängnisvoll. Wenn man also als Schlussfolgerung aus unseren Untersuchungen HsfA4 als antiapoptotischen Faktor einstuft, dann wäre HsfA5 der notwendige Gegenspieler. Es ist offensichtlich, dass diese aufregende biologische Dimension der vorgelegten Untersuchungen nur durch sogfältige Studien mit transgenen Pflanzen belegt oder auch widerlegt werden könnte. Die wesentlichen Ergebnisse aus diesen Untersuchungen wurden in Baniwal et al. J. Biol. Chem. 2007 (online seit 06. Dez. 2006) veröffentlicht.

7. REFERENCES

Almoguera, C., Rojas, A., Diaz-Martin, J., Prieto-Dapena, P., Carranco, R. Jordano, J. (2002) A seed-specific heat-shock transcription factor involved in developmental regulation during embryogenesis in sunflower. *J. Biol. Chem.* **277**: 43866-43872.

Ausubel, F., M., Brent, R., Kingston, R., E., Moore, D., D., Seidman, J., G., Smith, J., A., Struhl, K., (eds.) (1993) Current Protocols in Molecular Biology, John Wiley and Sons Inc, Indianapolis.

Baniwal, S., K., Bharti, K., Chan, K.,Y ., Fauth, M., Ganguli, A., Kotak, S., Mishra, S., K., Nover, L., Port, M., Scharf, K.-D., Tripp, J., Weber, C., Zielinski, D., von Koskull-Doring, P. (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *J. Biosci.* **29**: 471-87.

Baniwal, S., K., Chan, K., Y., Scharf, K.-D., Nover, L. (2007) Role of heat stress transcription factor HsfA5 as a specific repressor of HsfA4. *J. Biol. Chem.* **Dec 6**; [Epub ahead of print]

Bharti, K., Schmidt, E., Lyck, R., Bublak, D., Scharf, K.-D. (2000) Isolation and characterization of HsfA3, a new heat stress transcription factor of *Lycopersicon peruvianum*. *Plant J.* **22**: 355-365.

Bharti, K., von Koskull-Döring, P., Bharti, S., Kumar, P., Tintschl-Körbitzer, A., Treuter, E., Nover, L. (2004) Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with HAC1/CBP. *Plant Cell* **16**: 1521-1535.

Boscheinen, O., Lyck, R., Queitsch, C., Treuter, E., Zimarino, V., Scharf, K.-D. (1997) Heat stress transcription factors from tomato can functionally replace HSF1 in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **255**: 322-331.

Bukau, B., Weissman, J. Horwich, A. (2006) Molecular chaperones and protein quality control. *Cell* **125**: 443-451.

Busch, W., Wunderlich, M., Schoffl, F. (2005) Identification of novel heat shock factor-dependent genes and biochemical pathways in *Arabidopsis thaliana. Plant J.* **41**: 1-14.

Calderwood, S., K., Khaleque, M., A., Sawyer, D., B., Ciocca, D., R. (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem. Sci.* **31**: 164-172.

Cotto, J., J., Kline, M., Morimoto, R., I. (1996) Activation of heat shock factor 1 DNA-binding precedes stress-induced serine phosphorylation. *J. Biol. Chem.* **271**: 3335-3358.

Chan, K., Y., Baniwal, S., K., Nover, L., Scharf, K.-D. (2007) Manuscript in preparation.

Charng, Y., Y., Liu, H., C., Liu, N., Y., Chi, W., T., Wang, C., N., Chang, S., H., Wang, T., T. (2007) A Heat-inducible transcription factor, HsfA2, is required for

extension of acquired thermotolerance in Arabidopsis. *Plant Physiol.* **143**: 251-262.

Czarnecka-verner, E., Gurley, W., B., Nagao, R., T., Mosquera, L., A., Key, J., L. (1985) DNA sequence and transcript mapping of a soybean gene encoding a small heat shock protein. *Proc. Natl. Acad. Sci.* **82**: 3726-3730.

Czarnecka-Verner, E., Yuan, C.-X., Scharf, K.-D., Englich, G., Gurley, W., B. (2000) Plants contain a novel multi-member class of heat shock factors without transcriptional activator potential. *Plant Mol. Biol*. **43**: 459-471.

Davletova, S., Rizhsky, L., Liang, H., Shengqiang, Z., Oliver, D., J., Coutu, J., Shulaev, V., Schaluch, K., Mittler, R. (2005). Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen network of *Arabidopsis*. *Plant Cell* **17**: 268-281.

Döring, P., Treuter, E., Kistner, C., Lyck, R., Chen, A., Nover, L. (2000). The role of AHA motifs in the activator function of tomato heat stress transcription factors HsfA1a and HsfA2. *Plant Cell* **12**: 265-78.

Ellis, R., J., and Minton, A., P. (2006) Protein aggregation in crowded environment. *Biol. Chem.* **387**: 485-497.

Fu, S., Meeley, R., Scanlon, M., J. (2002) *Empty pericarp2* encodes a negative regulator of the heat shock response and is required for maize embryogenesis. *Plant Cell* **14**: 3119-3132.

Fu, S., Rogowsky, P., Nover, L., Scanlon, M., J. (2006) The maize heat shock factor-binding protein paralogs EMP2 and HSBP2 interact non-redundantly with specific heat shock factors. *Planta* **224**: 42-52.

Guettouche, T., Boellmann, F., Lane, W., S., Voellmy, R. (2005) Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochem.* **11**: 6-4

Hartl, F., U., Hayer-Hartl, M. (2002) Protein folding - Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**: 1852-1858.

Heerklotz, D., Döring, P., Bonzelius, F., Winkelhaus, S., Nover, L. (2001) The balance of nuclear import and export determines the intracellular distribution of tomato heat stress transcription factor HsfA2. *Mol. Cell Biol.* **21**: 1759-1768.

Hietakangas, V., Ahlskog, J., K., Jakobsson, A., M., Hellesuo, M., Sahlberg, N., M., Holmberg, C., I., Mikhailov, A., Palvimo, J., J., Pirkkala, L., Sistonen, L. (2003) Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol. Cell Biol.* **23**: 2953-2968.

Holmberg, C., I., Hietakangas, V., Mikhailov, A., Rantanen, J., O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R., I., Eriksson, J., E., Sistonen, L. (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *EMBO J.* **20**: 3800-3810.

Hong, Y., Sarge, K. D. (1999) Regulation of protein phosphatase 2A activity by heat shock transcription factor 2. *J. Biol. Chem*. **274**: 12967-12970.

Kotak, S., Port, M., Ganguli, A., Bicker, F., von Koskull-Döring, P. (2004) Characterization of C-terminal domains of *Arabidopsis* heat stress transcription factors (Hsfs) and identification of a new signature combination of plant class A Hsfs with AHA and NES motifs essential for activator function and intracellular localization. *Plant J.* **39**: 98–112.

Kotak, S., Vierling, E., Baumlein, H., von Koskull-Döring, P. (2007) A novel transcriptional cascade regulating expression of heat stress proteins during seed development of Arabidopsis. *Plant Cell* **Jan 12**; [Epub ahead of print]

Li, C., Chen, Q., Gao, X., Qi, B., Chen, N., Xu, S., Chen, J., Wang, X. (2005) AtHsfA2 modulates expression of stress responsive genes and enhances tolerance to heat and oxidative stress in *Arabidopsis*. *Sci. China C Life Sci.* **48**: 540-550.

Lyck, R., Harmening, U., Höhfeld, I., Scharf, K.-D. Nover, L. (1997) Identification of the nuclear localization signal of two tomato heat stress transcription factors. *Planta* **202**: 117-125.

Miller, G. and Mittler, R. (2006) Could heat shock transcription factors function as hydrogen peroxide sensors in plants. *Annals Bot.* **98**: 279-288.

Mishra, S., K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L., Scharf, K.-D. (2002) In the complex family of heat stress transcription factors HsfA1a has a unique role as master regulator of thermotolerance in tomato. *Genes Dev.* **16**: 1555-1565.

Morimoto, R., I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones and negative regulators. *Genes Dev*. **12**: 3788-3796.

Muratani, M., Tansey, W., P. (2003) How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* 4**:**192-201.

Nakai, A. (1999) New aspects in the vertebrate heat shock factor system: HsfA3 and HsfA4. *Cell Stress Chap.* **4**: 86-93.

Nishizawa, A., Yabuta, Y., Yoshida, E., Maruta, T., Yoshimura, K., Shigeoka, S. (2006) Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *Plant J.* **48**: 535–547.

Nover, L. (1987) Expression of heat shock genes in homologous and heterologous systems. *Enzyme Microb. Technol*. **9**: 130-144.

Nover, L., and Scharf, K.-D. (1997) Heat stress proteins and transcription factors. *Cell. Mol. Life Sci.* **53**: 80-103.

Nover, L., Bharti, K., Döring, P., Mishra, S., K., Ganguli, A., Scharf, K.-D. (2001) *Arabidopsis* and the heat stress transcription factor world: how many heat stress transcription factors do we need? *Cell Stress Chap*. **6**: 177-189.

Nover, L., Scharf, K.-D., Gagliardi, D., Vergne, P., Czarnecka-Verner, E., Gurley, W. B. (1996) The Hsf world: Classification and properties of plant heat stress transcription factors. *Cell Stress Chap*. **1**: 215-223.

Panchuk, I., I., Volkov, R., A., Schöffl, F. (2002) Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase

in *Arabidopsis*. *Plant Physiol*. **129**: 838-853.

Panikulangara, T., J., Eggers-Schumacher, G., Wunderlich, M., Stransky, H., Schöffl, F. (2004) Galactinol synthase 1, a novel heat-inducible and HSF-target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in *Arabidopsis*. *Plant Physiol*. **136**: 3148–3158.

Pelham, H., R., B., and Bienz, M. (1982) A synthetic heat-shock promoter element confers heat-inducibility on the Herpes simplex virus thymidine kinase gene. *EMBO J.* **1**: 1473-1477.

Pirkkala, L., Nykänen, P., Sistonen, L. (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.* **15**: 1118-1131.

Port, M., Tripp, J., Zielinski, D., Weber, C., Heerklotz, D., Winkelhaus, S., Bublak, D., Scharf, K.-D. (2004) Role of Hsp17.4-CII as coregulator and cytoplasmic retention factor of tomato heat stress transcription factor HsfA2. *Plant Physiol.* **135**: 1457-1470.

Ritossa, F. (1962) A new puffing pattern induced by heat shock and DNP in *Drosophila*. *Experientia* **18**: 571-573.

Rose, M., D., Winston, F., Hieter, P. (1990) Methods in Yeast Genetics. Cold Spring Harbor Lab. Press. Cold Spring Harbor, N. Y.

Sarge, K., D., Murphy, S., P., Morimoto, R., I. (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition

of DNA-binding activity, and nuclear localization and can occur in the absence of stress**.** *Mol. Cell Biol*. **13**:1392-1407.

Sarge, K., D., Zimarino, V., Holm, K., Wu, C., Morimoto, R., I. (1991) Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. *Genes Dev*. **5**:1902-1911.

Sambrook, J., Russell, D., W. (2001) Molecular cloning: A laboratory manual*.* 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Satyal, S., H., Chen, D., Fox, S., G., Kramer, J., M., Morimoto, R., I. (1998) Negative regulation of the heat shock transcriptional response by HSBP1. *Genes Dev.* **12**: 1962-1974.

Scharf, K.-D., Rose, S., Zott, W., Schöffl, F., Nover, L. (1990) Three tomato genes code for heat stress transcription factors with a region of remarkable homology to the DNA-binding domain of the yeast HSF. *EMBO J*. **9**:4495-4501.

Scharf, K.-D., Heider, H., Höhfeld, I., Lyck, R., Schmidt, E., Nover, L. (1998) The tomato Hsf system: HsfA2 needs interaction with HsfA1 for efficient nuclear import and may be localized in cytoplasmic heat stress granules. *Mol. Cell. Biol.* **18**: 2240-2251.

Scharf, K.-D., Höhfeld, I. Nover, L. (1998) Heat stress response and heat stress transcription factors. *J. Biosci*. **23**: 313-329.

Schöffl, F., Prändl, R., Reindl, A. (1998) Regulation of the heat-shock response. *Plant Physiol.* **117**: 1135-1141.

Schramm, F., Ganguli, A., Kiehlmann, E., Englich, G., Walch, D., von Koskull-Döring, P. (2006) The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in *Arabidopsis*. *Plant Mol. Biol.* **60**: 759-772.

Schuetz, T., J., Gallo, G., J., Sheldon, L., Tempst, P., Kingston, R., E. (1991) Isolation of a cDNA for HSF2: Evidence for two heat shock factor genes in humans. *Proc. Natl. Acad. Sci. USA* **88**: 6911-6915.

Shamovsky, I., Ivannikov, M., Kandel, E., S., Gershon, D., Nudler, E. (2006) RNA-mediated response to heat shock in mammalian cells. *Nature* **440**: 556-560.

Shi, Y., Mosser D., D., Morimoto, R., I. (1998) Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev.***12**: 654-666.

Somasundaram, T., and Bhat, S. P. (2004) Developmentally dictated expression of heat shock factors: exclusive expression of HSF4 in the postnatal lens and its specific interaction with alphaB-crystallin heat shock promoter. *J. Biol. Chem*. **279**: 44497-44503.

Sorger, P., K., and Pelham, H. R. (1988) Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54**: 855-864.

Töpfer, R., Schell, J. Steinbiss, H., H. (1988) Versatile cloning vectors for transient gene expression and direct gene transfer in plant cells. *Nucleic Acid Res.* **16**: 8725.

Treuter, E., Nover, L., Ohme, K., Scharf, K.-D. (1993) Promoter specificity and deletion analysis of three tomato heat stress transcription factors. *Mol. Gen. Genet.* **240**:113-125.

Voellmy, R. (2004) On mechanisms that control heat shock transcription factor activity in metazoan cells. *Cell Stress Chap.* **9**: 122-133.

Wiederrecht, G., Seto, D., Parker, C., S. (1988) Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell* **54**, 841-853.

Yamanouchi, U., Yano, M., Lin, H., Ashikari, M., Yamada, K. (2002) A rice spotted leaf gene, *Spl7*, encodes a heat stress transcription factor protein. *Proc. Natl. Acad. Sci. USA* **99**: 7530-7535.

Zou, J., Guo, Y., Guettouche, T., Smith, D., F., Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**: 471-480.

8. Appendix

SB2	pRT-3HA-HsfA2DBDxHsfA4bCTD	F-1111 and R-1112, T: tomato gDNA; amplicon cloned into PD241 via Sal*I* and Xba*I*.
SB3	pRT-3HA-LeHsfA5 (1-478)	F-1075 and R-1076, T: tomato cDNA; amplicon cloned into PD241 via Acc65*I* and Sal*I*.
SB4	pRT-3HA-HsfA2DBDxHsfB3CTD	F-1118 and R-1106, T: tomato gDNA; amplicon cloned into PD241 via EclX*I* and Xba*I*
SB5	pRT-HsfA2DBDxHsfA4bCTD	Acc65*I*-Xba*I* fragment from SB2 cloned into Acc65*I* and XbaI cut pRT-103.
SB12	pRT-3HA-HsfA2DBDxHsfA6CTD	Step 1) F-1193 and R-1114, T: tomato gDNA; amplicon cloned into pBSK^{+} via Sal*I* and Pst*I*. Step 2) Sal*I* and Not*I* fragment from the above plasmid was cloned into PD241 via Sal*I* and Not*I*.
SB13	pBSK^{+}-HsfA6; Intron (912 nucl.)	F-1113 and R-1114, T: tomato gDNA; amplicon cloned into pBSK^{+} via Xho*I* and Pst*I*.
SB14	pRT-HsfA6	XhoI and NotI fragment from SB13 cloned into pRT-103 via XhoI and NotI.
SB15	pBD-Gal4BD-AtHSBP1	F-187 and R-1261, T: At cDNA; amplicon cloned into bait vector (FB5) via EcoR*I* and Not*I*.
SB16	pRT-3HA-HsfA2DBDxHsfA5CTD	F-1252 and R-1260, T: SB3; amplicon cloned into PD241 via Sal*I* and Not*I*. **Note**: This clone has NVGQST amino acid residues extra as a result of cloning procedure.
SB17	pRT-3HA-HsfA2DBDxHsfA5CTD	F-270 and R-1259, T: pRT-HsfA2; and F-1252 and R-1260, T: SB3. Cloned into PD241 via triple ligation Acc65*I* –Sal*I*-Not*I*.
SB19	pRT-3HA-HsfA2.8xHsfA5.8	F-270 and R-414, T: ET720; F-1254 and R-1260, T: SB3. Cloned into PD241 via triple ligation Acc65*I* –Sal*I*-Not*I*.
SB24	pRT-3HA-HsfA2.7xHsfA5.7	F-270 and R-414, T: ET711; F-1253 and R-1260, T: SB3. Cloned into PD241 via triple ligation Acc65*I* –Sal*I*-Not*I*.
SB25, 26, 27, 36, 37, 50	pEntry- AtA4a; AtA4c; AtA5, LeA4b, LeA5, AtHSBP1,	Sal*I*-Not*I* fragments from SZ21, SZ26, AG10, SB2, SB17, SB15 repectively cloned into pENTRY® (invitrogen) via Sal*I*-Not*I*.
SB28 and 30	pRT-3HA: HSBP1 from tomato and At	F-1197and R-1198, T: tomato cDNA; F-1195 and R-1196, T: At cDNA. Respective amplicons were cloned into PD241 via Acc65*I* and Ecl*XI*.
SB29 and 31	pGEX: HSBP1from tomato and At	F-1197 and R-1198, T: tomato cDNA; F1195 and R-1196, T: At cDNA. Respective amplicons were cloned into pGEX2-GST (RC21, Bharti et al. 2004) via Acc65*I* and Ecl*XI*.
SB33	pRT-3HA-HsfA5DBDxHsfA2CTD	F-270 and R-1255, T: SB3; F-1258 and R-535, T: pRT-HsfA2; Cloned into PD241 via triple ligation Acc65*I*-Sal*I*-Not*I*.
SB34	pRT-3HA-HsfA5.7xHsfA2.7	F-270 and R-1256, T: SB3; F-272 and R-535, T: pRT-HsfA2.7 (ET711); Cloned into PD241 via triple ligation Acc65*I*-Sal*I*-Not*I*.
SB35	pRT-3HA-HsfA5.8xHsfA2.8	F-270 and R-1257, T: SB3; F-272 and R-535, T: pRT-HsfA2.8 (ET720); Cloned into PD241 via triple ligation Acc65*I*-Sal*I*-Not*I*.
SB38, 39, 40, 41	pJawohl8-RNAi: CTD from Hsfs AtA5, AtA4a, AtA4c, LeA5	Sal*I* and Not*I* fragments from respective pENTRY® (invitrogen) clones (i.e. SB27, SB25, SB26 and SB37).
SB42 43, 44, 45, 46, 47, 48, 51, 53, 54,56	pRT-3HA-HsfA5DBDx- CTD from Hsfs AtA1a, AtA1b, AtA1e, AtA2, AtA3, AtA4c, AtA7a, LeA4b, LpA1a, LpB1, AtA5(W439A),	Sal*I* and Not*I* fragments from respective pRT clones (i.e. AG1, AG5, SZ25, AG8, SZ20, SZ26, AG7, SB8, KB241, KB240, MP80) were cloned into SB20 via Sal*I* and Not*I*.
SB49	pBD-Gal4BD-LeHsfA5CTD	Sal*I* and Not*I* fragment from SB17 was cloned into pBD (modified by M.Port) via Sal*I* and Not*I*.
SB55	pRT-LeA2DBDxAtA5CTD (W439A)	Sal*I*-Not*I* fragment from MP80 was cloned into SB17 via Sal*I* and Not*I*.
SB57	pRT-3HA-AtHsfA5(W439A)	Xba*I* fragment from MP80 was cloned into AG10 via Xba*I*.
SB58	pRT-3HA-LeHsfA4b(18-393)	F-1428 and R-1392, T: SB79; Cloned into SB2 via triple ligation Acc65*I*-Sal*I*.
SB60	pAD5d-3HALpA2DBD-A4bCTD	Xho*I*-Xba*I* fragment from SB2 was cloned into pAD5Δ via Sal*I* -Avr*II*.

SB62	pRTdS-N-Strep	Synthesized double stranded oligo with cutting sites for KpnI, SalI and SacI SB2 was cloned into MK33 via Nco*I*-Sac*I*.
SB63	pGal4AD-AtHSBPI	Sal*I*–Not*I* fragment from SB15 was cloned into MP59 via Sal*I*-Not*I*.
SB64, 65, 66, 67	pRT-LeA5DBDxLeA2CTD, LeA5.7xLeA2.7, LeA5.8xLeA2.8, LpA2DBDxLeHsfA4bCTD	Acc65*I*-Xba*I* fragments from SB33, SB34, SB35, SB2 repectively were cloned into SB61 via Acc65*I*-Xba*I*.
SB68, 69, 70, 71, 72, 73, 85	pRTdS-Strep LpA2DBDxLeHsfA4bCTD, LeA5DBDxLpA2CTD, LeA5.7NTDxLpA2.7 LeA5.8xLpA2.8, LpA1, LpA2, LeA4b	Acc65*I*-Xba*I* fragments from SB2, SB33, SB34, SB35, pRT-3HA-LpA1 LS, pRTdS-3HA-LpA2, SB79 repectively were cloned into SB62 via Acc65*I*-Xba*I*.
SB74, 75, 76, 77, 78, 87	pRTdS-Strep- Hsfs- AtA4a, AtA4c, AtA5, AtA5(W439A), LeA5, AtA3	Acc65*I*-Not*I* fragments from AG26, AG13, AG19, SB57, SB3, AG12 repectively were cloned into SB70 via Acc65*I*- Not*I*.
SB79	pRT-3HA-LeHsfA4b	F-1135 and R-1392, T: Le Sepal cDNA; Cloned into SB2 via Acc65*I*-Sal*I*.
SB80, 84, 97, 98, 99	pAD5Δ-Strep-LeA5.8xLpA2.8, LpA2DBDxLeA4bCTD, 3HA-LeA4b, AtA4c, LeA4b-Strep	Nco*I*-Xba*I* fragments from SB71, SB67, SB79, SB75, SB88 were respectively cloned into pAD5Δ via Nco*I*-Avr*II*.
SB81, 82, 83, 145	pAD5d-Strep-AtA5, AtA5(W439A), AtA4a, 3HA-LeA5	Nco*I*-Not*I* fragments from SB76, SB77, SB74, SB3 respectively were cloned into SB80 via Nco*I*-Not*I*.
SB88	pRTdS-LeA4b-Strep	F-1580 and R-1581, T: SB79; Cloned into pRTdS-Strep (C-term) via Nco*I*-Nhe*I*.
SB92	pRT-3HALeA5(Δ:OD)	Sal*I*-Not*I* fragment from SB19 were cloned into SB21 via Sal*I*-Not*I*.
SB94	pBD-LeA5 (W429A)	F---- and R-179, T: SB3; Cloned into pBD (modified by M. Port) via Acc65*I*-Sal*I*.
SB95	pRTdS-Strep-AtA5DBD(M)	F-894 and R-1603, T: SB76; Cloned into SB 70 via Acc65*I*-Not*I*. (Mega-primer strategy was used)
SB100	pRT-3HA-LeA5.8(SalI)	Sal*I*-Not*I* fragment from SB19 was cloned into SB35 via Sal*I*-Not*I*.
SB105	pRT-3HA-Gal4BDxLeA5CTD	Sal*I*-Not*I* fragment from SB17 was cloned into pRT-3HA-Gal4BD via Sal*I*-Not*I*.
SB106	pRT-3HA-LeA5(Δ:281-426)	Bgl*II*-Not*I* fragment from SB143 was cloned into SB131 via Bgl*II*-Not*I*.
SB107	pRT-3HA-LeA5(Δ:336-426)	Bgl*II*-Not*I* fragment from SB143 was cloned into SB132 via Bgl*II*-Not*I*.
SB111	pRTdS-LeA4b-3HA	Nhe*I*-Xba*I* fragment from pRT-AtHsfB1-3HA was cloned into SB88 via Nhe*I*-Xba*I*.
SB112	pRTdS-LeA4b-Myc	Nhe*I*-Nde*I* fragment from pRT-AtHsp18.5-Myc (M. Siddique) was cloned into SB111 via Nhe*I*-Nde*I*.
SB119	pRT-3HA-LeHsfA5-LS	Not*I*-Ssp*I* fragment from HsfA1-LS was cloned into SB3 via Not*I*-Ssp*I*.
SB120	pRT-3HA-LeA5(1-239)	Xba*I* fragment was digested out from SB3 and the rest of the plasmid was self ligated.
SB127	pRT-3HA-LeA5(BglII-A)	Tripple ligation: F-270 and R-1835/ F-1834 and R-179, T: SB3; Cloned into SB3 via Acc65*I*-Bgl*II*-Nhe*I*.
SB128	pRT-3HA-LeA5(BglII-C)	Tripple ligation: F-270 and R-1839/ F-1838 and R-179, T: SB3; Cloned into SB3 via Acc65*I*-Bgl*II*-Nhe*I*.
SB129	pRT-3HA-LeA5(BglII-B)	Tripple ligation: F-270 and R-1835/ F-1836 and R-179, T: SB3; Cloned into SB3 via Acc65*I*-Bgl*II*-Nhe*I*.
SB130	pRT-3HA-LeA5(Δ:Box A)	Tripple ligation: F-1254 and R-1835/F-1838 and R-1260, T- SB3; cloned into SB3 via Cla*I*-Bgl*II*-Nhe*I*.
SB131	pRT-3HA-LeA5(Δ:Boxes A and B)	Tripple ligation: F-1254 and R-1835/F-1836 and R-1260, T- SB3; cloned into SB3 via Cla*I*-Bgl*II*-Nhe*I*.
SB132	pRT-3HA-LeA5(Δ:Box B)	Tripple ligation: F-1254 and R-1839/F-1836 and R-1260, T- SB3; cloned into SB3 via Cla*I*-Bgl*II*-Nhe*I*.
SB133	pJC-3HA-LeA5-6His	F-1763 and R-1765, T- SB3; cloned into pJC-HsfB1CTD-6His (Bharti et al. 2004) via Xho*I*-Apa*I*.
SB135	pRT-3HA-LeA5(OD:HsfA1)	F-618 and R-645, T- pRT-HsfA1 (Bharti el al. 2004); cloned into SB92 via Sal*I*.
SB136 137	pGST- Strep-AtA5, LeA4b-3HA	Nco*I*-Xba*I* fragments from SB76, SB111 were cloned respectively into FS93 i.e. pGST-AtHsfA2 via Nco*I*-Xba*I*.
SB138	pRT-3HA-LeA5 (1-446)-LS	SB119 i.e. pRT-3HA-LeA5 LS was Nhe*I* cut and the plasmid re-circularized.

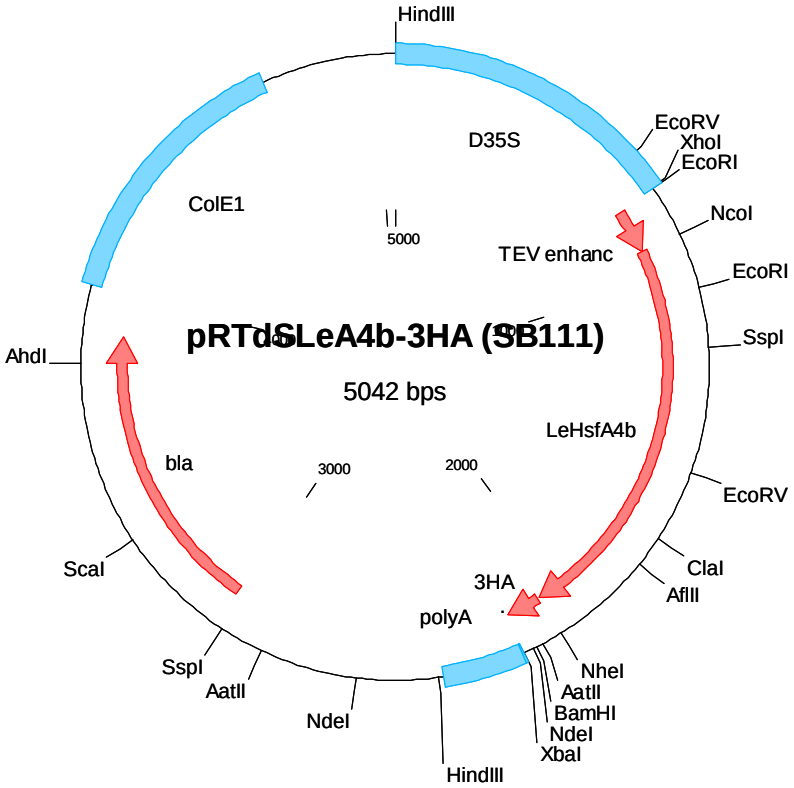
SB139, 140, 141	pRT-3HA- LeA5 (1-278), (1-333), (1-375)	SB127, 128, and 129 were cut with Bgl*II*-Not*I* and the plasmids were end-filled using Klenov reaction DNA Polymerase-I (large fragment) and re-circularized.
SB142	pRT-3HA-LeA5(Δ:410-426)	Tripple ligation: F-270 and R-1903/F-1904 and R-179, T- SB3; cloned into SB129 via Acc65*I*-Bgl*II*-Not*I*.
SB143	pRT-3HA-LeA5(Δ:378-426)	F-1904 and R-179, T- SB3; cloned into SB129 via Bgl*II*-Not*I*.
SB146	pHub1-Gal4BD	Yeast two hybrid bait vector with obtained from Shravan K. Mishra at MPI, Munich.
SB147	pMBI-3HA-LeA5	Sal*I* fragment from SB145 was cloned into pMBI7 via Sal*I*.
SB148	pRTdS-GST-LeA4bCTD	Sal*I*-EcoR*I* fragment from SB111 was cloned into pRTdS-GST (from M. Siddique) via Sal*I*-EcoR*I*.
SB149, 150	pRTdS-GST-LeA4bCTD-Strep, LeA4bCTD-3HA	Afl*II*-Xba*I* fragments from SB88, SB111 were respectively cloned into SB148 via Afl*II* and Xba*I*.
SB151, 152	pMBI-LeA4b (-3HA, Strep)	Nco*I*-Xba*I* fragments from SB111, SB88 were respectively cloned in to SB147 via Nco*I*-Avr*II*.
SB153, 154	pRT-3HA-LeA5(1-446)xAD from: Gal4 (768-881), VP-16 (413-490)	Nhe*I*-Ssp*I* fragments from KB315, KB316 (Bharti et al. 2004) were respectively cloned in to SB3 via Nhe*I*-Ssp*I*.
SB155	pRTdS-Strep-3HA-LeA5	Sal*I* fragment from SB147 was cloned into SB62 via Sal*I*.
SB156	pRT-3HA-LeA5DBDxVP-16AD	Sal*I*-Not*I* fragment from MP39 was cloned into SB33 via Sal*I*-Not*I***.**
SB157	pRTdS-3HA-Gal4BDxLeA5CTD	Nco*I* fragment from SB105 was cloned into SB155 via Nco*I***.**
SB159	pRTdS-3HA-Gal4BD	SB157 was cut with Sal*I* and plasmid was re-circularized.
SB160	pRTdS-3HA-LeA5(1-446)	Xba*I* fragment from SB138 was cloned into SB155 via Xba*I***.**
Sb162	pRTdS-3HA-Gal4BDxLeA5(110-478, W429A)	Xba*I* fragment from SB94 was cloned into SB157 via Xba*I***.**
SB163	pRTds-3HA-LeA5(1-446)xVP-16AD(413-490)	Xba*I* fragment from SB154 was cloned into SB155 via Xba*I***.**
SB164	pRTds-3HA-Gal4BDxLeA5(110-446)xVP-16AD(413-490)	Xba*I* fragment from SB154 was cloned into SB157 via Xba*I***.**
SB165	pET26b-LeA4b-Strep	Nco*I*-Sac*I* fragment from SB99 was cloned into pET-26b(+) via Nco*I*-Sac*I*.
SB168	pRTdS-Strep-Myc-LeA5(1-236)	F-2304 and R-179, T- SB3; cloned into SB169 via Sac*I*-Xba*I*.
SB169	pRTdS-Strep-Myc-LpA2	Xho*I*-Xba*I* fragment from MK34 was cloned into SB62 via Xho*I*-Xba*I***.**
SB170	pRTdS-Strep-Myc-A2BDxA5CTD	Nde*I* fragment from SB17 was cloned into SB169 via Nde*I***.**
SB171	pGST-3HA-LeA5-6His	Nco*I*-Hind*III* fragment from SB133 was cloned into SB137 via Nco*I*-Hind*III***.**
SB172	pGST-LeA4b-Strep	Nco*I*- Xba*I* fragment from SB88 was cloned into SB137 via NcoI-Xba*I*.
SB174, 175	pRTdS-A5DBDxA4bCTD –Myc/3HA	F-270 and R-1255, T- SB168; cloned into SB112/SB111 via Nco*I*-Sal*I*.
SB176	pRTdS-3HA-LeA5CTD(110-478)	F-2558 and R-179, T- SB3; cloned into SB157 via Acc65*I*-Sal*I*.
SB177	pGST-3HA-LeA5CTD(110-478)	Acc65*I*-Xba*I* fragment from SB176 was cloned into SB171 via Acc65*I*-Xba*I***.**
SB178	pRTdS-3HA-LeA5(110-235)	SB176 was Xba*I* cut and the plasmid was self ligated**.**
SB179	pRTdS-GFP-LeA5	Tripple ligation (Sac*I*-Xba*I*-Xba*I*): Sac*I* and Xba*I* fragment from SB168 and Xba*I* fragment from SB155 were cloned into pRTdS-GFP- LpA2**.**
SB180	pRT-GFP-LeA5(1-330)	Sac*I*-Xba*I* fragment from SB168 was cloned into pRTdS-GFP- LpA2 via Sac*I*-Xba*I*.
SB183	pRT-LpA1(OD:HsfA4b)	F-1642 and R-1645, T- SB111; cloned into pRTLpA1Δ7/8 via Sal*I*.
SB184	pRTdS-3HA-LeA5(110-200)	F-2552 and R-2588, T- SB3; cloned into SB178 via Acc65*I*-Xba*I*.
SB185	pRT-dS-GFP-LeA4b-3HA	F-2598 and R-179, T- SB111; cloned into pRTdS-GFP-DCP2 (from Christian Weber) via Bgl*II*-Xba*I*.
SB186	pRTdS-Strep-LeA4b (18-393)-Strep	Asp718-Xba*I* fragment from SB58 was cloned into SB168 via Asp718-Xba*I*.
SB187	pJC-LpA1(OD:A4b)	Sac*I*-Apa*I* from SB183 was cloned into pJC-HsfA1 (Bharti et al. 2004) via Sac*I*-Apa*I*.
SB188	pGST-3HA-LeA5(110-200)	Acc65*I*-Xba*I* fragment from SB184 was cloned into SB171 via Asp718-Xba*I***.**
SB189	pJC40-LeA4b-3HA	Xho*I*-Xba*I* fragment from SB111 was cloned into MK38 i.e. pJC40-10xHis-PsHsp18.1(I) via Xho*I*-Xba*I*.

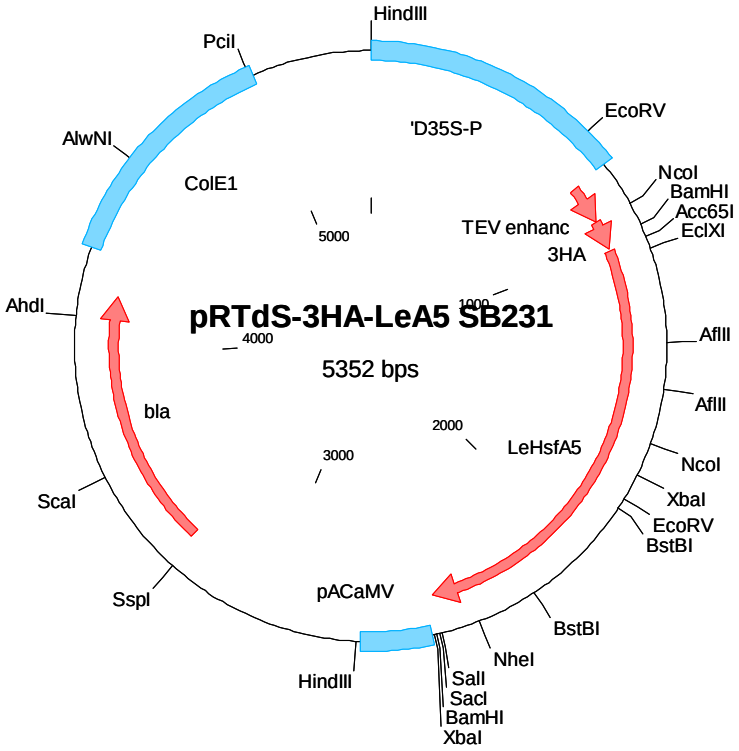
SB190	pRTdS-3HA-LeA5(200-478)	F-2601 and R-179, T- SB3; cloned into SB176 via Acc65*I*-Xba*I*.
SB191, 196	pRTdS-3HA-LeA5-Myc-Yn, Yc	Tripple ligation: F-2601 and R-1835, T- SB3 (Pag*I*-Bgl*II*)/ F-1834 and R-2602, T- SB3 (Bgl*II*-Xba*I*); cloned into pRTds-Myc-Yn, pRTds-Myc-Yc via Nco*I* and Nhe*I*.
SB192, 193	pRTdS-LeA4b-3HA-Myc-Yn, Yc	Nco*I*-Nhe*I* fragments from SB111 were cloned into pRTds-Myc-Yn, pRTds-Myc-Yc via Nco*I* and Nhe*I*.
SB194	pRTdS-3HA-LeA5(200-478)	F-2626 and R-179, T- SB3; cloned into SB176 via Acc65*I*-Xba*I*.
SB195	pJC40-3HA-LeA5	Tripple ligation (Nco*I*/Pag*I*-Nhe*I*/Xba*I*): F-2601 and R-1835, T- SB3/ F-1834 and R-2602, T- SB3; cloned into MK38 i.e. pJC40-10xHis-PsHsp18.1 via Nco*I*-Nhe*I*.
SB197	pJC40-3HA-LeA5(Δ:OD)	Acc65*I*-Nhe*I* fragment from SB92 was cloned into SB195 via Acc65*I*-Nhe*I*.
SB198	pJC40-3HA-LeA5(OD: HsfA1)	Acc65*I*-Nhe*I* fragment from SB135 was cloned into SB195 via Acc65*I*-Nhe*I*.
SB199	pJC40-3HA-LeA5(110-478)	Acc65*I*-Nhe*I* fragment from SB176 was cloned into SB195 via Acc65*I*-Nhe*I*.
SB200	pJC40-3HA-LeA5(200-478)	Acc65*I*-Nhe*I* fragment from SB190 was cloned into SB195 via Acc65*I*-Nhe*I*.
SB201	pJC40-3HA-LeA5(Δ:HR-A)	Acc65*I*-Nhe*I* fragment from SB194 was cloned into SB195 via Acc65*I*-Nhe*I*.
SB202	pGST-LeA5(ΔOD)	Acc65*I*-Nhe*I* fragment from SB92 was cloned into SB171 via Acc65*I*-Nhe*I*.
SB203	pGST-LeA5(A1: HR-AB)	Acc65*I*-Nhe*I* fragment from SB135 was cloned into SB171 via Acc65*I*-Nhe*I*.
SB204	pGST-LeA5(200-478)	Acc65*I*-Nhe*I* fragment from SB190 was cloned into SB171 via Acc65*I*-Nhe*I*.
SB205	pRTdS-3HA-LeA5(Δ:OD)-Yn	Acc65*I*-Nhe*I* fragment from SB92 was cloned into SB191 via Acc65*I*-Nhe*I*.
SB206	pRTdS-CFP-LeA4b-3HA	Nco*I*-Nhe*I* fragment from SB185 was cloned into pRTdS-Dcp1-CFP (CW78) via Nco*I*-Nhe*I*.
SB207	pMAL-c2X	Vector plasmid obtained from New England BioLabs England (NEB).
SB208	pMAL-c2X-HA-LeA5	BamH*I*-Sal*I* fragment from SB155 was cloned into SB207 via BamH*I*-Sal*I*.
SB209	pMAL-c2X-HA-LeA4b	BamH*I*-Sal*I* fragment from SB79 was cloned into SB207 via BamH*I*-Sal*I*.
SB210	pRTdS-LeA4b(Δ:OD)-3HA	Tripple ligation (Nco*I*-Sal*I*-Nhe*I*): F-1580 and R-1641, T- Le (Sepal) cDNA/ F-1644 and R-179, T- SB111; cloned into SB111.
SB211	pRT-3HA-LeA5(OD: A4b)	F-1645(Sal*I*) and R-1642(Xho*I*), T- SB111; cloned into SB92 via Sal*I*.
SB212	pMAL-c2X-HA-LeA5(110-200)	BamH*I*-Xba*I* fragment from SB184 was cloned into SB207 via BamH*I*-Xba*I*.
SB213	pMAL-c2X-HA-LeA5(Δ:OD)	Acc65*I*-Nhe*I* fragment from SB92 was cloned into SB208 via Acc65*I*-Nhe*I*.
SB214	pMAL-c2X-HA-LeA5(A1: HR-AB)	Acc65*I*-Nhe*I* fragment from SB135 was cloned into SB208 via Acc65*I*-Nhe*I*.
SB215	pRTdS-3HA-LeA5(OD:A4b)-Myc-Yn	Afl*II*-Nhe*I* fragment from SB211 was cloned into SB191 via Afl*II*-Nhe*I*.
SB216	pRTdS-3HA-LeA5(OD:A4b)-HA-Yc	Afl*II*-Nhe*I* fragment from SB211 was cloned into SB196 via Afl*II*-Nhe*I*.
SB217	pMAL-c2X-HA-LeA4b-Strep	F-2706 and R-179, T- SB88; cloned into SB207 via BamH*I*-Xba*I*.
SB219	pRTdS-LeA5(OD:A4b)	Acc65*I*-Nhe*I* fragment from SB211 was cloned into SB176 via Acc65*I*-Nhe*I*.
SB220	pMAL2cX-LeA4b(d: HR-A/B)-Strep	Nco*I*-Nhe*I* fragment from SB210 was cloned into SB217 via Nco*I*-Nhe*I*.
SB221	pRTdS-LeA4b(Δ:OD)-Myc-Yn	Nco*I*-Nhe*I* fragment from SB210 was cloned into pRTds-Myc-Yn via Nco*I*-Nhe*I*.
SB222	pRTdS-LeA4b(Δ:OD)-HA-Yc	Nco*I*-Nhe*I* fragment from SB210 was cloned into pRTds-HA-Yn via Nco*I*-Nhe*I*.
SB223	pRTdS-LeA4b(OD:LeA5)	F-1253(Sal*I*) and R-1257(Xho*I*), T- SB3; cloned into SB210 via Sal*I*.
SB224	pRTdS-LeA4b(OD:LpA1)	F-618 and R-645, T- pRT-LpA1; cloned into SB210 via Sal*I*.
SB225	pRTdS-GFP-LeA4b(Δ:OD)-3HA	Ssp*I* fragment from SB210 was cloned into SB185 via Ssp*I*.
SB226	pMAL-c2X-HA-LeA5(110-478)	Acc65*I*-Nhe*I* fragment from SB176 was cloned into SB208 via Acc65*I*-Nhe*I*.
SB227	pMAL-c2X-HA-LeA5(200-478)	Acc65*I*-Nhe*I* fragment from SB190 was cloned into SB208 via Acc65*I*-Nhe*I*.
SB228	pMAL-c2X-LpA1	EcoR*I* fragment from pRT-LpA1.LS was cloned into SB207 via EcoR*I*.
SB230	pRTdS-GFP-LeA5(1-446)	EclX*I*-Ssp*I* fragment from SB138 was cloned into SB179 via EclX*I*-Ssp*I*.
SB231	pRTdS-3HA-LeA5	Acc65*I*-Nhe*I* fragment from SB3 was cloned into SB176 via Acc65*I*-Nhe*I*.
SB233	pRTdS-LeA5(ΔOD)-Yc	Acc65*I*-Nhe*I* fragment from SB92 was cloned into SB196 via Acc65*I*-Nhe*I*.
SB234	pRTdS-LeA5(OD:A1)-Yc	Acc65*I*-Nhe*I* fragment from SB135 was cloned into SB196 via Acc65*I*-Nhe*I*.

SB235	pRTdS-LeA5(OD:A4b)-Yc	Acc65*I*-Nhe*I* fragment from SB211 was cloned into SB196 via Acc65*I*-Nhe*I*.
SB236	pRTdS-GFP-LeA5(ΔBox-A)	Nco*I*-Nhe*I* fragment from SB130 was cloned into SB179 via Nco*I*-Nhe*I*.
SB237	pRTdS-GFP-LeA5(ΔBox-A and B)	Nco*I*-Nhe*I* fragment from SB131 was cloned into SB179 via Nco*I*-Nhe*I*.
SB238	pRTdS-GFP-LeA5(ΔBox-B)	Nco*I*-Nhe*I* fragment from SB132 was cloned into SB179 via Nco*I*-Nhe*I*.
SB239	pRTdS-3HA-AtA4a	Acc65*I*-Xba*I* fragment from SB74 was cloned into SB231 via Acc65*I*-Xba*I*.
SB240	pRTdS-3HA-AtA4c	Acc65*I*-Xba*I* fragment from SB75 was cloned into SB231 via Acc65*I*-Xba*I*.
SB241	pRTdS-3HA-AtA5	Acc65*I*-Xba*I* fragment from SB76 was cloned into SB231 via Acc65*I*-Xba*I*.
SB242	pRTdS-3HA-AtA5(R77D)	Acc65*I*-Xba*I* fragment from SB95 was cloned into SB231 via Acc65*I*-Xba*I*.
SB243	pRTdS-3HA-LeA5(R72D)	Same strategy as was used for constructing SB95 (F-1603 was used at low stringency conditions PCR)
SB245	pMAL-HA-LeA5 (R72D)	BamH*I*-Sal*I* fragment from SB243 was cloned into SB207 via BamH*I*-Sal*I*.

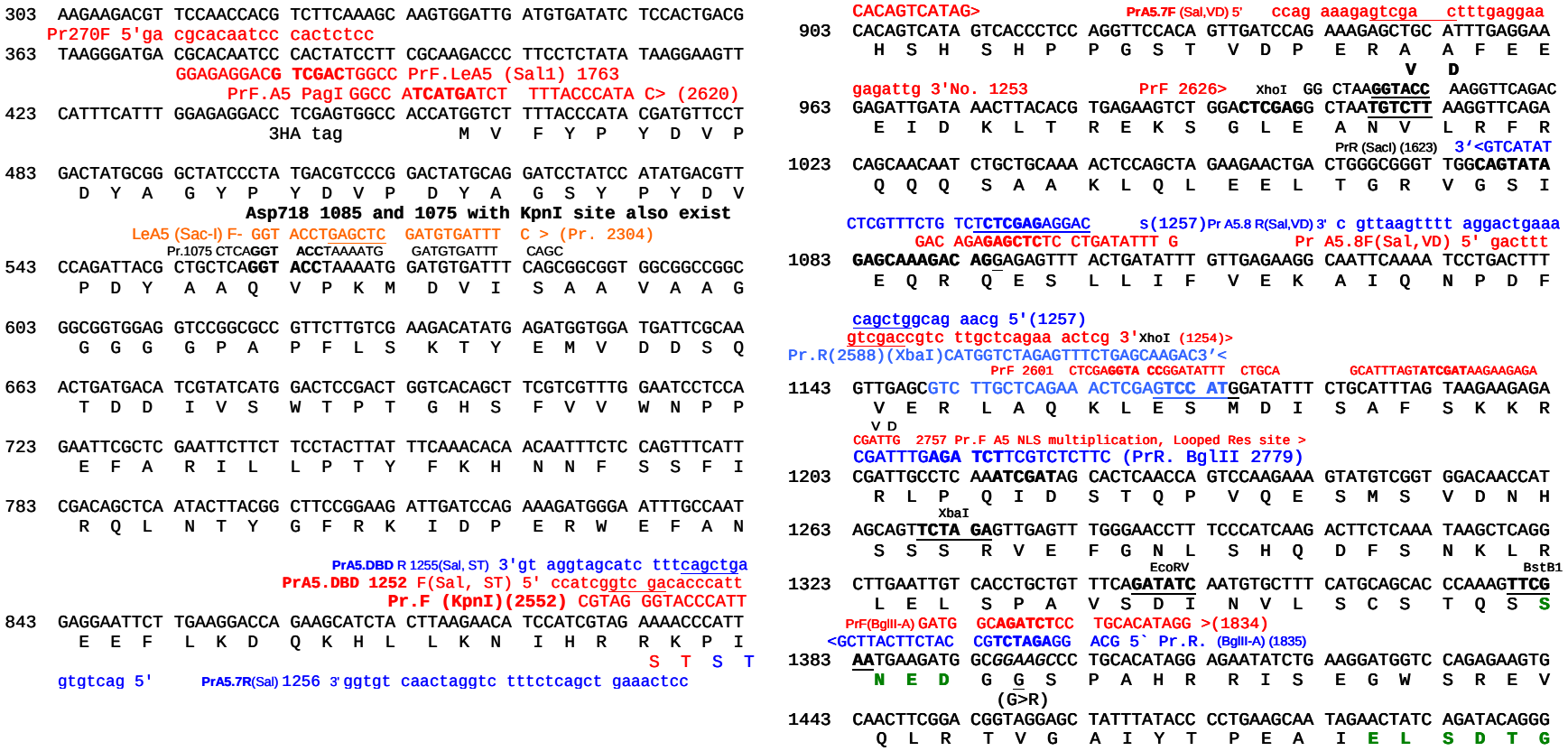
Template DNA *i.e.* Genomic (gDNA) or complimentary (cDNA), for PCR were always synthesized from leaves unless specified.

Standard plasmid maps *e.g.* pRTdS-LeHsfA4b and pRTdS-3HALeHsfA5





Sequence of LeHsA4b-3HA and the available PCR primers



Sequence of 3HA-HsfA5 and the available PCR primers

```
1   GAAGTTCATT TCATTTGGAG AGGACCTCGA GTGGCCACCA TGGTCTTTTA CCCATACGAT GTTCCTGACT ATGCGGGCTA TCCCTATGAC
                                  (3HA Tag) M  V  F   Y  P  Y  D   V  P  D   Y  A  G   Y  P  Y  D
                                   PrFStA4b(KpnI) (1135)  5‘-AATAGGGTACCAGTGTACATGGTAAGCATAGTTATGG-3‘ >
                                                                    Pr.F(NcoI)  5’GCATAGC CATGGATAAC
                                                                  PrF(2598)(Bgl-II) 5’GATAGC
                                                                Pr2706F LeA4bF-BamHI  CGAACGGGATCC ATGGATAAC
 91   GTCCCGGACT ATGCAGGATC CTATCCATAT GACGTTCCAG ATTACGCTGC TCAGGTACCA GTGTACATGG TAAGCATAGT TATGGATAAC
       V  P  D   Y  A  G   S  Y  P  Y   D  V  P   D  Y  A   A  Q  V  P   V  Y  M   V  S  I   V  M  D  N

    Pr.F (Kpn1) 5’  G  GATCAAGTTCTTCGGTACCGGCGCC > No-1428
        TGTAATGG> Pr. 1580
        AGATCTTAACTGTAATGG>(to be used on SB111)
        TG> Pr2706 (use on SB88 or 111)
181   TGTAATGGAG GATCAAGTTC TTCTTCTCCG GCGCCTTTTT TGTTGAAAAC TTATGAACTG GTTGATGATT CGTATACTAA TCCAGTTGTT
       C  N  G   G  S  S   S  S  S  P   A  P  F   L  L  K   T  Y  E  L   V  D  D   S  Y  T   N  P  V  V

                                                          EcoRI
271   TCATGGAGCC ATAACGGACG TAGCTTCGTT GTTTGGAATC CACCTGAATT CGCTAGAGAT TTGCTTCCGA AATACTTTAA GCATAACAAT
       S  W  S   H  N  G   R  S  F  V   V  W  N   P  P  E   F  A  R  D   L  L  P   K  Y  F   K  H  N  N

361   TTCTCAAGTT TTATCAGACA ACTTAATACT TAT GTAAGTA AAATTATTTC GTAAAAATAT CTCTATCGAA TCATCACCAA TTGTGTTTTG
       F  S  S   F  I  R   Q  L  N  T   Y                                                (Intron 88 nucl.)

451   TTATTTGACG ATATTCATTT ATTTCTATTA G GGGTTTAGA AAGGTTGATC CTGAACAATG GGAGTTTGCG AACGAGGATT TTTTAAGA
                                        G  F  R   K  V  D   P  E  Q   W  E  F  A   N  E  D   F  L  R
                              Pr.R1392  AG  ACGAAAGTCGACCCATAGC (Sal1)  5’
                                        5’CGA AAGTCGACCCATAGCCACTCTGC> Pr.F 1111 (Sal1)  PrF XhoI TG TAGCTCCATT
                                                                                < PrR 1641 (SalI VD) TG TAGCTCCATT
   GGACGTAGACAT TTGTTGAAGA ATATTTATAG ACGAAAGCCG ATCCATAGCC ACTCTGCTGC AGCAGGAACA GGGCAATCTG TAGCTCCATT
     G  R  R  H   L  L  K   N  I  Y   R  R  K  P   I  H  S   H  S  A   A  A  G  T   G  Q  S   V  A  P

     GCTCGAGTCTGAGAGAC (1642)>
     GGTCGACTCT GAGAGAC
631   GACGGATTCT GAGAGACAGG AGTATGAAGA TGAAATCGAG AGGTTGAAGA GAGAAAACAG TCTTCTTCAG TCGTCTGCGG AGAATCAATT
      L  T  D  S   E  R  Q   E  Y  E   D  E  I  E   R  L  K   R  E  N   S  L  L  Q   S  S  A   E  N  Q

721   GAAATTCAAT GGGGAGTACG AAAGTGGAAT TAAGTCTATG GAGCAACGTT TACAGAACGT TGCTCATAGA CAGGGGAAAT TGATTTCTCT
      L  K  F  N   G  E  Y   E  S  G   I  K  S  M   E  Q  R   L  Q  N   V  A  H  R   Q  G  K   L  I  S
```

```
                                    < 5'CTGCTCGCGCTGTCGACGAAATCAGATG   PrR SalI(1645)
                                  Pr.F (1644)CATCTGATTTCGTCGACAGCGCGAGCAG>      NLS
 811  TTTAGCTCAA TTACTACAAA CACCTGGATT TTCATCTGAT TTCGCTCAAA GCGCGAGCAG GAAGAGACGA TTGTTGATAT CAAATTACTT
      L  L  A  Q   L  L  Q   T  P  G   F  S  S  D   F  A  Q   S  A  S   R  K  R  R   L  L  I   S  N  Y

 901  GATTGACGAG GAAAACTCAC CAAAATTCGA CTTGGAAATG GTTAAAAAGT TGGATTCATC AATCAATTTT TGGGAGCGGT TTCTGTATGG
      L  I  D  E   E  N  S   P  K  F   D  L  E  M   V  K  K   L  D  S   S  I  N  F   W  E  R   F  L  Y
                                                                                                    ClaI
 991  TGTTCAAACA CAAGATTTCG AGCATACACA TTCCCCAATT GTTACACATA CATCATCTAA TGATTCTGCC AAACGAAACT CTCCCATCGA
      G  V  Q  T   Q  D  F   E  H  T   H  S  P  I   V  T  H   T  S  S   N  D  S  A   K  R  N   S  P  I
                                                                                      AflII
1081  TCATTCACCA TCCTCCTCCG AGTTAGGGCC ATTGAATCCT GTCATGTCAT CAACTTATGA AAATTTAGAA CGTCAACTTA AGCCATCTGA
      D  H  S  P   S  S  S   E  L  G   P  L  N  P   V  M  S   S  T  Y   E  N  L  E   R  Q  L   K  P  S
                                                                          AatII
                                                 Pr 1826 F>5' GGTAATGA CGTCGCGGCG CAACAGTTCT TAACAGAG 3'
1171  TAATCAGATT GAGTGTAAGA CCAGTAAAAC ATCTGAATTA GTATCAAACT CGGGTAATGA TGTATTTTGG CAACAGTTCT TAACAGAGAC
      D  N  Q  I   E  C  K   T  S  K   T  S  E  L   V  S  N   S  G  N   D  V  F  W   Q  Q  F   L  T  E
                                                                          A  A

1261  GCCTGGTTGC ACTGAGCCAC AACAAGTTGA GAACAAAGGG ATAAACGAAT CAACGCGTGA TATTAGATTA GGGGATAGCC ATAGATATTG
      T  P  G  C   T  E  P   Q  Q  V   E  N  K  G   I  N  E   S  T  R   D  I  R  L   G  D  S   H  R  Y

                                                                    Pr.R (Xba1) (1112) < GTCAAATCTAGAA

                                                                Pr.R (NheI) 1581  < GCAACAGGA AGCGCTAGCTGATTC
                                                                                    GG TCTAATGACCCGGGATCTCAGG
1351  GTGGAATCGC GGAGTTAATT TAGAAAATCT TGCTGAAAGA ATGGGACATC TTAGTAGTCC AGCAACAGGA AGCTGAGTTG ATTCTAGAGT
      W  W  N  R   G  V  N   L  E  N   L  A  E  R   M  G  H   L  S  S   P  A  T  G   S  -
      TCAACTCAGCTTCC (from EST seq so no corresponding seq in the plasmid)
1441  CCGCAAAAAT CACCAGTCTC TCAGCTCAAT TACTACAAAC ACCTGGATTT CATCTGATTT CGCTCAAGCG CGAGCAGGAG AGACGATGTT
 <Pr.R 1214(NotI-XbaI)GACATT TTCTAGAGTCCGCGGCCGCC ACC

1531  GATATCAATA CTTGATGACG
```

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PUBLICATIONS

- **S. K. Baniwal**, K. Y. Chan, K.-D. Scharf, L. Nover.
  Role of heat stress transcription factor HsfA5 as specific repressor of HsfA4.
  J. Biol. Chem., (Dec 2006) 10.1074/jbc.M609545200.

- **S. K. Baniwal**, K. Bharti, K. Y. Chan, M. Fauth, A. Ganguli, S. Kotak, S. K. Mishra, L. Nover, M. Port, K.-D. Scharf, J. Tripp, C. Weber, D. Zielinski, P. von Koskull-Döring (2004).
  Heat stress response in plants: A complex game with chaperones and more than twenty heat stress transcription factors. *J. Bioscience*, 29 (4): 471-487.

- S. S. Yazdani, A. R. Shakri, P. Mukherjee, **S. K. Baniwal**, C. E. Chitnis (2004).
  Evaluation of immune responses elicited in mice against a recombinant malaria vaccine based on *Plasmodium vivax* Duffy binding protein. *Vaccine*, 22 (27-28): 3727-37.

STATEMENT (ERKLÄRUNG)

Ich erkläre hiermit, daß ich die Diplomarbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Frankfurt am Main, den 22.01.07

Sanjeev K. Baniwal