Protein interactions of heat stress transcription factors from *Lycopersicon peruvianum*

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 15
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
Raffaella Calligaris
aus Trieste, Italien

Frankfurt im Mai 2001

Vom Fachbereich .....15..... der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan: Prof. Dr. Karl-Dieter Entian

Gutachter: Prof. Dr. Lutz Nover
Prof. Dr. Heinz Rüterjans

Datum der Disputation: 25 Oktober 2001

Index

Acknowledgements

Abbreviations

aa	Amino acid residue
AD	Activation domain
A_x	Absorbance (at wavelength x nm)
AHA	Activator motifs of Hsfs with aromatic, large hydrophobic and acidic amino acid residues
At-	*Arabidopsis thaliana*
3-AT	3-Amino-1,2,4-triazole
BD	Binding domain
BSA	Bovine serum albumin
C	Control condition during the heat stress regime
CHO-K1 cells	Chinese hamster ovary cell culture
DBD	DNA-binding domain
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ECL	Enhanced chemiluminescence
EGS	Ethyleneglycol-bis (sucinic acid N-hydroxysuccinimide) ester
EMSA	Electrophoretic mobility shift assay
FOA	Fluoro-orotic acid
GST-tag	Glutathione S-transferase-tag
HeLa	Human epithelial cell culture derived from a cervical carcinoma
HEPES	N-2-hydroxyethyl piperazine-N´-2-ethanesulfonic acid
HIS3	Imidazolyl glycerol phosphate dehydratase
His_6-tag	6 Histidine-tag
HR-A/B	Hydrophobic heptad repeats in region A and B of OD
HR-C	C-terminal hydrophobic heptad repeat
HS	Heat stress
HSE	Heat stress element
Hsf	Heat stress transcription factor
HSGs	Heat stress granules
Hsps	Heat stress proteins
HR	Hydrophobic region
kDa	kiloDalton
IPTG	Isopropyl-β-D-thiogalactopyranoside
Lp-	*Lycopersicon peruvianum*
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MCS	Multiple cloning site
MS	Mass spectrometry
m/z	Mass-to-charge ratio
NES	Nuclear export signal
NLS	Nuclear localization signal
Nonidet-P40	Polyethylenglycol-p-isooctylphenylether-P40
NTA	Nitrilotriacetic acid
OD	Oligomerization domain
OD_x	Optical density (at wavelength x nm)

P.....	Preinduced condition during the heat stress regime
PCR.....	Polymerase chain reaction
Pefabloc.....	4-(2-Aminoethyl)-benzolsufonylfluorid
pI.....	Isoelectric point
Ps-.....	*Pisum sativum*
PVDF.....	Polyvinylidene difluoride
Sc-.....	*Saccharomyces cerevisiae*
SDS.....	Sodium dodecyl sulfate
SDS-PAGE.....	SDS-polyacrylamide gel electrophoresis
sHsp.....	small heat stress proteins
TCA.....	Trichloroacetic acid
TFA.....	Trifluoroacetic acid
Tris.....	Tris-(Hydroxyl)-methylamin
v/v.....	Volume/volume
U.....	Unit
WCE.....	Whole cell extract
w/v.....	Weight/volume

One-letter and three-letter codes for the 20 amino acid residues:

A.....	Ala	Alanine	M.....	Met	Methionine
C.....	Cys	Cysteine	N.....	Asn	Asparagine
D.....	Asp	Aspartic acid	P.....	Pro	Proline
E.....	Glu	Glutamic acid	Q.....	Gln	Glutamine
F.....	Phe	Phenylalanine	R.....	Arg	Arginine
G.....	Gly	Glycine	S.....	Ser	Serine
H.....	His	Histidine	T.....	Thr	Threonine
I.....	Ile	Isoleucine	V.....	Val	Valine
K.....	Lys	Lysine	W.....	Trp	Tryptophan
L.....	Leu	Leucine	Y.....	Tyr	Tyrosine

1 Introduction

1.1 The heat stress response

Cells of all organisms respond to heat and other forms of stress (oxidative stress, bacterial infection, etc.) by triggering a global transition in gene expression.

Typically, the expression of most genes is either shut down or greatly reduced and a specific group of genes, called heat stress (HS) genes, is rapidly induced to high levels (Schlesinger et al., 1982) (Fig. 1.1). This phenomenon, the heat stress response, was discovered by Ritossa (1962), who observed an induction of specific puffs on the polytene chromosomes of *Drosophila melanogaster* salivary glands upon heat or chemical treatment. Subsequent studies elucidated the nature of the induced mRNAs and proteins at the molecular level and led to the isolation and characterization of the HS genes (reviewed by Nover et al., 1989a; Wu, 1995; Scharf et al., 1998a). Proteins encoded by the HS genes, the heat stress proteins (Hsps), enable cells to survive harmful conditions by preventing and repairing damages caused to other proteins. The HS response is transient and during stress conditions Hsps expression is regulated by evolutionary conserved DNA-binding proteins called heat stress transcription factors (Hsfs).

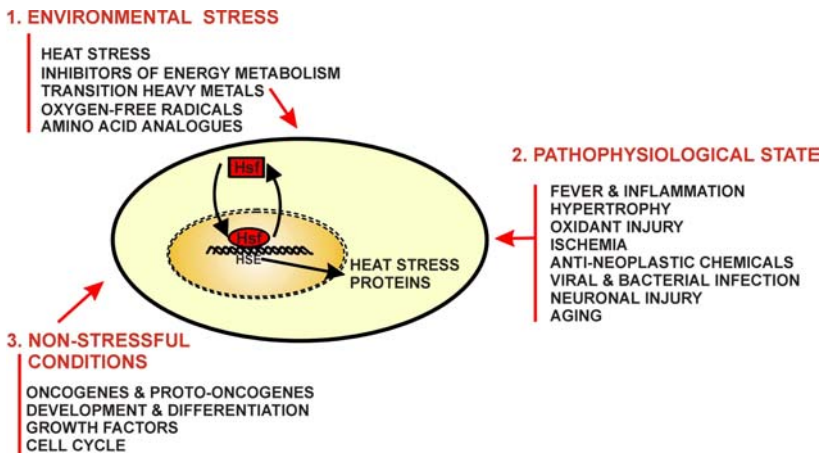


Figure 1.1 Inducers of the cellular stress response. Representation of three general classes of conditions (environmental stress, pathophysiological state and non-stressful conditions) known to result in the elevated expression of heat stress proteins. Heat stress genes expression is resulting by the activation of Hsf which binds to the heat stress elements (HSEs). (modified from Morimoto, 1998).

1.2 The heat stress proteins (Hsps)

Most members of the Hsp family are constitutive, heat inducible and ubiquitously expressed. On the basis of their size and inducibility, Hsps have been classified into multiprotein families (reviewed by Nover and Scharf, 1997), which include Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps (Fig. 1.2). The latter are the most diverse and abundant Hsps synthesized by plants (Waters et al., 1996).

Plant small Hsps (sHsps) have monomeric molecular masses in the range of 15 to 25 kDa, they form oligomeric complexes, and share a characteristic carboxyl-terminal domain with signature amino acid motifs (DeJong et al., 1993, Waters et al., 1996). The sHsps can be divided into at least five classes: two classes are found in the cytoplasm (class I and II) and the other three are organelle-localized (mitochondria, plastids and endoplasmatic reticulum) (Waters et al., 1996; Scharf et al., 2001).

The conservation of Hsps between bacteria and eukaryotic organisms suggests an ancient function that was essential for survival throughout evolution. Indeed, in the past decade it has been shown that many of the Hsps have essential functions as multiprotein chaperone machines involved not only in protection against stress damage but also in protein folding, topogenesis, translocation and degradation, as well as in signal transduction cascades (Hartl, 1996; Morimoto et al., 1994; Parsell and Lindquist, 1993; Vierling, 1991; Forreiter and Nover, 1998).

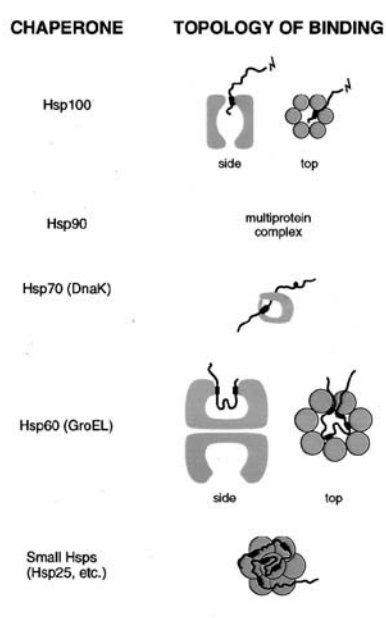


Figure 1.2 Major classes of Hsps. The chaperone activity (action) of the indicated Hsps (chaperone) is reported as well as the topology of the action (topology of binding). Bold lines signify polypeptides and the thickened segments denote sites that become directly associated with chaperone, typically hydrophobic in character. Structures are not drown to scale. Relevant references are indicated. (modified from Bukau and Horowich, 1998)

1.3 The heat stress transcription factors (Hsfs)

Hsfs are transcriptional regulators of the heat stress response. They are activated by a multitude of stresses (Fig. 1.1) and they induce the expression of many heat stress-responsive genes (Wu, 1995). Promoters of genes controlled by Hsfs contain DNA sequence motifs, called heat stress elements (HSEs), which are composed of multiple, inverted, tandem repeats of the consensus sequence AGAAn, where 'n' can be any nucleotide (Amin et al., 1988; Xiao and Lis, 1988; Nover et al., 1989a).

Hsfs belong to a multigene family: number and overall sequence vary widely among different species. Unlike yeast and *Drosophila*, which appear to have only one Hsf gene (Wiederecht et al., 1988; Clos et al., 1990), most vertebrates have multiple Hsf genes: at least three genes have been isolated from human, mouse and chicken (Rabindran et al., 1991; Schuetz et al., 1991; Sarge et al., 1991; Nakai and Morimoto, 1993; Nakai et al., 1997). The Hsf multiplicity is even higher in plants: four genes have been identified in tomato (Scharf et al., 1990 and 1993; Bharti et al., 2000), six in soybean (Czarnecka-Verner at al., 1995) and twenty-one in *Arabidopsis* (Hübel and Schöffl, 1994; Nover et al., 2001). This multiplicity is further enhanced by the presence of isoforms of the same protein (Fiorenza et al., 1995; Goodson et al., 1995) and by the stress-dependent expression of some of these (Scharf et al., 1990; reviewed by Nover and Scharf, 1997). The latter is a peculiarity of plants.

Like other transcription factors, Hsfs share a common arrangement of structural and functional modules: a N-terminal DNA-binding domain, an adjacent oligomerization domain, a nuclear localization signal and a carboxyl-terminal transactivation domain (Fig. 1.3 A). The details of Hsfs structure have been nicely reviewed by Wu (1995), Nover et al. (1996), Nover and Scharf (1997) and Scharf et al. (1998a). The main features are described in the following paragraphs with special reference to plant Hsfs. In particular, on the basis of structural evolutionary homology, a unified nomenclature for plant Hsfs has been developed (Nover et al., 1996 and 2001), which results in the

assignment of Hsfs to class A or class B (Fig. 1.3 B). The main criteria for this classification are:

- analysis of the DNA-binding domain
- mode of expression (constitutive vs. heat stress induced)
- length and structure of the L1 and L2 linker regions
- fine structure of the oligomerization domain (HR-A/B region)
- position of a cluster of basic amino acid residues (K/R motif or NLS)

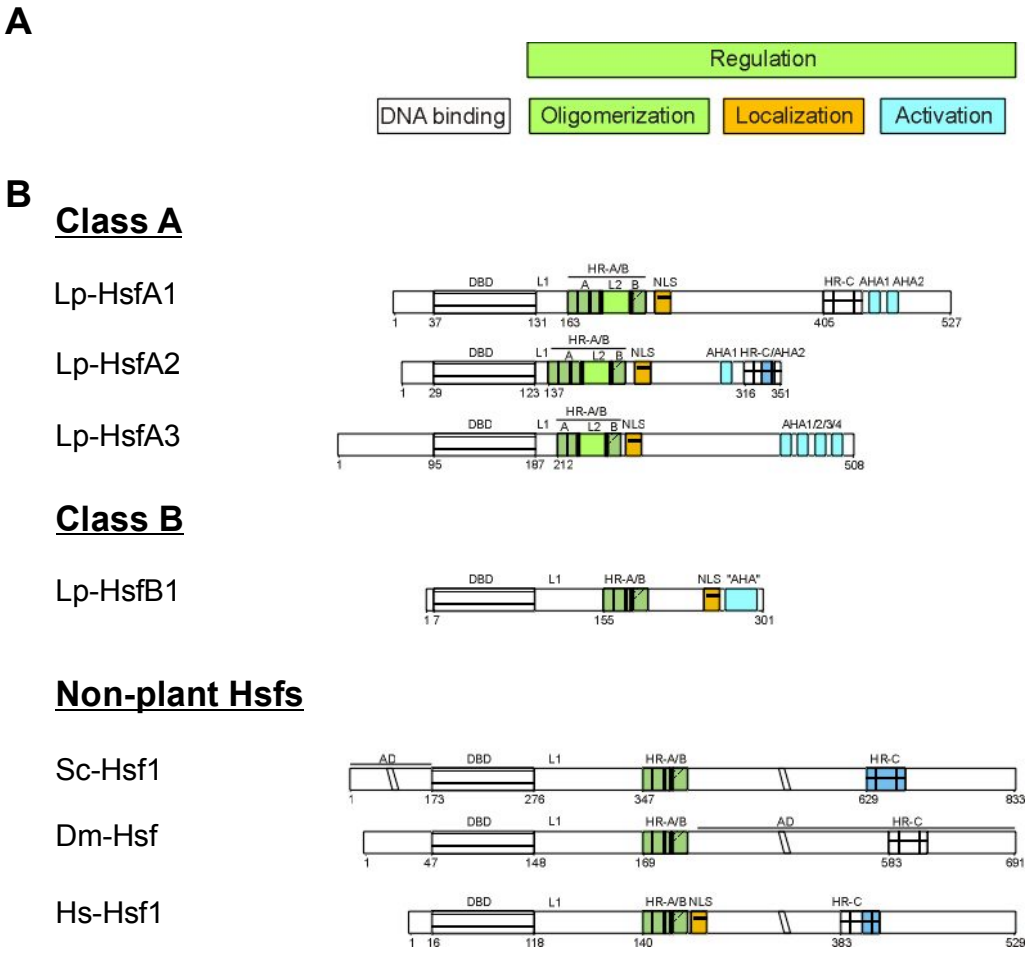


Figure 1.3 Basic structure of Heat stress transcription factors (Hsfs). (**A**) The common functional modules of the structure of Hsfs are depicted in block diagrams. (**B**) Schematic representation of HsfA1, HsfA2, HsfA3 and HsfB1 from tomato. For comparison, non-plant Hsfs are also shown. DBD: DNA-binding domain, HR-A/B and HR-C: hydrophobic heptad repeat regions; NLS: nuclear localization signal; AD: activation domain; L1: linker of variable length; L2: linker absent in plant class B and non-plant Hsfs; AHA: Aromatic, large hydrophobic and acidic amino acid residues. Lp: *Lycopersicon peruvianum*; Sc: *Saccharomyces cerevisiae*; Dm: *Drosophila melanogaster*; Hs: *Homo sapiens.*

1.3.1 The DNA-binding domain (DBD)

This N-terminal region of about 100 amino acids is the most highly conserved and well studied domain among Hsfs from different species. The crystal and solution structures of the DBD of Hsf from *Kluyveromyces lactis* (Damberger et al., 1994; Harrison et al.,1994), as well as the solution structure of *Drosophila melanogaster* (Vuister et al., 1994) and *Lycopersicon peruvianum* (Schultheiss et al., 1996) DBDs, show that Hsfs are members of the winged Helix-Turn-Helix (HTH) family of DNA-binding proteins. This tightly packed globular structure is formed by a three-α–helical bundle on one side and a four-stranded antiparallel β-sheet on the other side. The third helix (α3), which forms a classical helix with polar and positively charged residues exposed to the solvent, has been described as the helix responsible for HSE recognition (Schultheiss et al., 1996; Wu, 1995). In particular the lack of an unstructured 11-12 residues motif between the β3- and β4- strands is the major difference between plant and non-plant Hsfs. Thus, in plants, the β3- and β4-strands are connected by a turn of only four amino acid residues (Nover and Scharf, 1997). A flexible linker of variable length connects the C-terminus of the β4-strand of DBD to the oligomerization domain.

1.3.2 The oligomerization domain (HR-A/B)

This domain is characterized by a series of hydrophobic heptad repeats (HR) consisting of large hydrophobic amino acids, which suggest a coiled-coil structure characteristic of leucine-zipper type protein interaction domains (Landschulz et al., 1988; Lovejoy et al., 1993). The heptad repeats occur in two regions (HR-A and HR-B), connected by a flexible linker (Sorger and Nelson, 1989, Rabindran et al., 1993; Zuo et al., 1994 and 1995). Plant class A Hsfs present a characteristic insertion of 21 amino acid residues in the linker, which give rise to a second heptad repeat pattern (open circles and stars, Fig. 1.4). On the contrary, plant class B Hsfs and all non plant Hsfs do not have this insertion and present a single and continuous heptad repeat pattern (full circles, Fig. 1.4). Other amino acid residues, beside those in the heptad position, are

highly conserved or invariant in the HR-A/B region, suggesting that they contribute to the specificity of the interactions mediated by this domain.

The relevance of this domain is not only in determining the trimerization of the Hsfs but also in their regulation and function (Jakobsen and Pelham, 1991; Chen et al., 1993; Zuo et al., 1994). For example, the result of trimerization is the presence of three binding domains, which largely increase the affinity for DNA-binding sites (Drees et al., 1997).

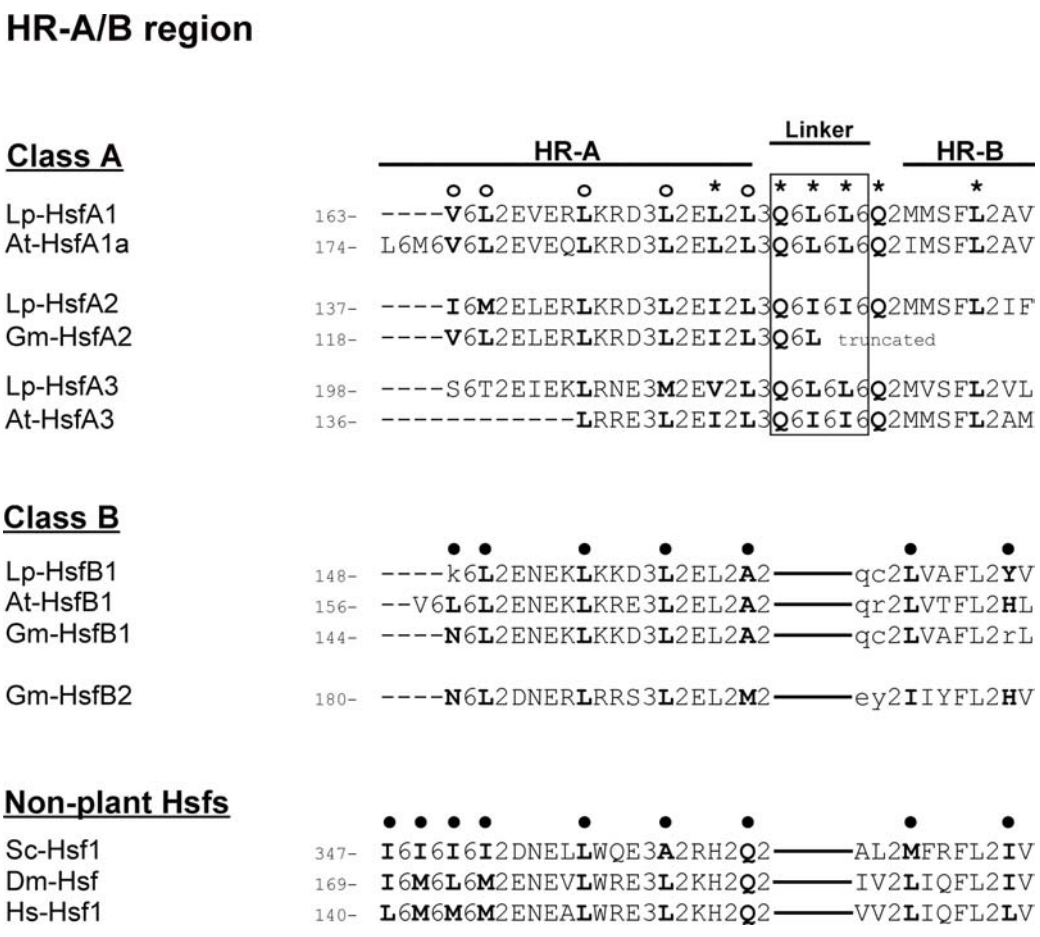


Figure 1.4 Sequence comparison of the HR-A/B region of oligomerization domains from different Hsfs. Protein sequences are labeled with protein name to the left. Numbers denote the first amino acid residue in each sequence. The heptad repeat pattern may extend beyond the indicated position, e.g. in At-Hsf1 or in non-plant Hsfs. The heptad repeat positions are marked: two overlapping repeat patterns are found in class A Hsfs (open circles and stars respectively), whereas all other have a single continuous pattern (closed circles). Highly conserved residues which are not in frame with the repeats are marked by shading. The insert of 21 amino acid residues (Linker) of the class A Hsfs is boxed. 'truncated': sequence is incomplete at the C-terminus. Lp: *Lycopersicon peruvianum*; At: *Arabidopsis thaliana*; Gm: *Glycine max*; Sc: *Saccharomyces cerevisiae*; Dm: *Drosophila melanogaster*; Hs: *Homo sapiens.*

1.3.3 The nuclear sequence motifs (NLS and NES)

All Hsfs contain two clusters of basic (Lys and Arg) amino acid residues, the K/R motifs, considered as putative nuclear localization signals (NLS). According to a sequence motif comparison both clusters are NLS of the bipartite type (Robbins et al., 1991). K/R1 represents the conserved C-terminal part of the DBD, whereas K/R2 is present in the C-terminal domain either close to the HR-A/B region or more distal in class B Hsfs. An exception is the unique NLS of the yeast Hsf (*S. cerevisiae*), which is positioned at the C-terminus and is a NLS of the monopartite type. Attempts to investigate the role of each K/R motif in human Hsf led to the conclusion that both K/R motifs (Sheldon and Kingston, 1993) or only the K/R1 motif (Zuo et al., 1995) are required for nuclear import. Studies with mutants of tomato HsfA1 and HsfA2 have shown that only K/R2 functions as NLS (Lyck et al., 1997) and that a deletion of the K/R2 motif in HsfB1 results in a protein defective in nuclear import (unpublished results from our group).

The nuclear export of Hsfs is less studied. To date, the leucine-rich NES (nuclear export signal) at the C-terminus of tomato HsfA2 is the only export signal identified in any Hsf (Heerklotz et al., 2001).

1.3.4 The C-terminal hydrophobic heptad repeat (HR-C)

In addition to the HR-A/B region of the oligomerization domain, a third conserved domain (HR-C) is localized close to the C-terminus of many Hsfs. This heptad repeat consists of only few hydrophobic amino acids and therefore may not be able to maintain the stable protein interactions typical of leucine-zippers. Mutations and the complete deletion of this region in human, *Drosophila* and plants lead to constitutive activation and deregulation of Hsfs expression (Lyck et al., 1997; Nakai and Morimoto, 1993; Rabindran et al., 1993; Sheldon and Kingston, 1993; Zuo et al., 1994 and 1995). These findings led to a general model for the regulation of Hsf trimerization and DNA-binding (Fig. 1.5). It proposes that under non-stressful conditions the HR-C region would interact with the HR-A/B region, thus shielding the oligomerization

domain and keeping the factor in an inactive, monomeric form (Lis and Wu, 1993). Furthermore, the intramolecular interaction between the HR-C and the HR-A/B would mask the NLS and inhibit the nuclear import.

1.3.5 The activation domain (AD)

This C-terminal region shows a very low degree of sequence conservation and can be considered as an acidic activation domain, enriched in proline residues in most Hsfs from vertebrates, *Drosophila* and plants. Interestingly plant class B Hsfs are an exception because of a neutral or positively charged domain (i.e. glutamine or proline rich residues are absent); moreover they are mostly known to be without transcriptional activator potential (Czarnecka-Verner et al., 2000).

In general the AD is characterized by partly redundant elements, which are involved in the Hsfs function and regulation (Nieto-Sotelo et al., 1990; Nakai and Morimoto, 1993; Rabindran et al. 1993; Hoj and Jakobsen, 1994; Shi et al., 1995; Zuo et al., 1995; Wisniewski et al., 1996; Lyck et al., 1997). The essential functional elements of tomato class A Hsfs have been analyzed by deletion and point mutations and have been defined as short peptide motifs (AHA motifs) with a characteristic pattern of aromatic (A) and large hydrophobic (H) amino acid residues situated in an acidic (A) context (Treuter et al., 1993; Döring et al., 2000). These motifs reside in or close to the HR-C region presenting a central tryptophan residue as an essential element for the activator function and are assumed to be the putative interacting sites with components of the basal transcription machinery (Döring et al., 2000; Bharti et al., 2000).

1.4 Multiple functions of Hsfs

A number of studies indicate that the stress-induced Hsps expression is not the only function of Hsfs. For example, the *hsf* gene is essential for viability of yeast under non-stress conditions (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Gallo et al., 1993). Indeed, a yeast cell cycle mutant blocked in G2

that maps to the *hsf* gene has been identified, indicating that some functions of the Hsf are important for cell cycle progression (Smith and Yaffe, 1991). In addition, deletion of the *hsf* gene in *D. melanogaster* results in defects in oogenesis and early larval development, indicating that these defects are not due to altered basal expression of Hsps but rather to some other as yet unknown targets or functions of the Hsf (Jedlicka et al., 1997).

The existence of multiple Hsfs in all other species suggests that the various Hsfs might have more specialized functions in response to distinct physiological and environmental stimuli. Among the three known mammalian Hsfs, Hsf1 is the major form expressed in all cells and, beside being a regulator of the stress response under pathophysiological conditions, is also required for extraembryonic development and postnatal growth (Baler et al., 1993; Sarge et al., 1993; Xiao et al., 1999). Hsf2 has been suggested to have a role during development (Schuetz et al., 1991; Sistonen et al., 1992; Sarge et al., 1994; Mezger et al., 1994; Alastalo et al., 1998; Pirkkala et al., 1999) and Hsf4 lacks the usual properties of a transcriptional activator and its overexpression in HeLa cells results in decreased expression of endogenous Hsp genes (Nakai et al., 1997). In avian cells, Hsf1 and HsfA3 are heat stress responsive and independently regulated; in fact, cells lacking Hsf3 are severely compromised for induction of the heat stress response even though Hsf1 is expressed. (Nakai et al., 1995; Tanabe et al., 1998).

1.5 Protein-protein interactions regulate Hsfs activity

The activation of Hsfs relies on many protein-protein interactions (Fig. 1.5) that directly or indirectly contribute to two major changes: an increase in DNA-binding affinity to the heat stress element sequences (HSEs) and the acquisition of transcription-stimulatory activity. These events have been mostly studied in yeast and vertebrates and indicate many Hsf-specific peculiarities.

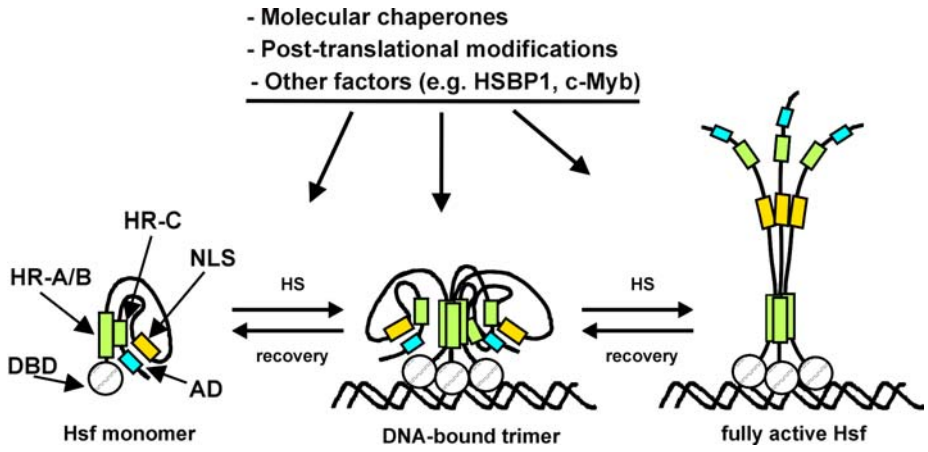


Figure 1.5 Model of Hsfs regulation in higher eukaryotes. Schematic representation of monomer-to-trimer transition of Hsfs. This transition and in general Hsfs activity are regulated by protein-protein interaction given by intra- and intermolecular coiled-coil interactions, post-translational modifications (e.g. phosphorylation and ubiquitination), molecular chaperones (Hsp70, Hsp90 and cochaperones) and other factors (e.g. HSBP1, c-Myb and p53). (modified from Wu, 1995)

1.5.1 Intra- and intermolecular interactions of Hsfs

In higher eukaryotes under non-stressful conditions, intramolecular interactions between amino- and carboxyl-terminal coiled-coil domains are thought to prevent Hsf monomer from assuming the active trimeric form (Chen et al., 1993; Rabindran et al., 1993; Zuo et al., 1994). As already mentioned (1.3.4) the carboxyl-terminal hydrophobic repeat (HR-C) is required to repress trimerization of Hsf1 in human cells at physiological temperatures and a similar requirement was found for *Drosophila* Hsf (Rabindran et al., 1993). Furthermore, deletions or substitutions of hydrophobic residues in either the HR-C or the oligomerization (HR-A/B) domain caused constitutive oligomerization and DNA-binding activity of Hsf (Zuo et al., 1994; Farkas et al., 1998). Instead, under stressful conditions, three Hsf molecules interact via the oligomerization domain to form homotrimers with strong DNA-binding activity (Perisic et al., 1989; Sorger et al., 1989).

A conformational change also represents a change in the nature of the cooperative interactions between subunits within an Hsf trimer and between adjacent trimers to HSEs. The part of the protein critical for these cooperative

DNA-binding activities has been localized to the DNA-binding and trimerization domains (Fernandes et al., 1995; Kroeger et al., 1994; Littlefield and Nelson, 1999).

1.5.2 Post-translational modifications

Targeted post-translational modifications also appear to play a critical role in modulating Hsfs activities; phosphorylation has been particularly studied.

A number of studies have revealed that protein kinases, like ERK1/ERK2 (Knauf et al., 1996), and phosphatases, like CDC2a (Reindl et al., 1997) and PP2A (Hong and Sarge, 1999), physically interact with Hsf. Thus, Hsf is moderately phosphorylated on serine and threonine residues under non-heat-stress conditions and becomes hyperphosphorylated upon heat stress (Cotto et al., 1996; Sorger et al., 1988). Several studies have demonstrated that for yeast, *Drosophila* and human Hsf alteration in phosphorylation levels probably plays no role in regulating trimerization or DNA-binding (Cotto et al., 1996; Hoj and Jakobsen, 1994; Knauf et al., 1996; Xia et al., 1998). Nevertheless, a work suggests that, *in vitro*, phosphorylation of *Arabidopsis* Hsf by the cyclin dependent kinase CDC2a leads to a decrease in DNA-binding affinity (Reindl et al., 1997). Other experiments suggest that phosphorylation of Hsf may play a role in deactivation of transcriptional competence. The hyperphosphorylation of *K. lactis* Hsf is involved in returning Hsf to the inactive state after heat stress (Hoj and Jakobsen, 1994), whereas human Hsf activity is repressed by constitutive moderate level of phosphorylation (Kline and Morimoto, 1997; Knauf et al., 1996) and enhanced by hyperphosphorylation (Xia and Voellmy, 1997). However, the heat stress induced dephosphorylation of a specific amino acid residue in Hsf1 may be directly responsible for increased transcription (Xia et al., 1998).

Recent findings also report regulation of some Hsfs (i.e. human Hsf2) by the ubiquitin-proteosome pathway (Mathew et al., 1998).

1.5.3 Hsfs and the transcription machinery complex

DNA-bound Hsf must communicate with the transcription machinery through protein contacts. Indeed, TBP (TATA-box binding protein) was found to interact with *Drosophila* Hsf in vitro (Mason and Lis, 1997) and with *Arabidopsis* Hsf *in vitro* and *in vivo* (Reindl and Schöffl, 1998).

In the context of Hsf-DNA interaction and transcription, noticeable is the fact that Hsf associates with components of the chromatin remodeling machinery (Becker and Wu, 1992; Tsukiyama et al., 1994; Brown and Kingston, 1997). Recently a direct physical interaction has been shown between tomato class A Hsfs and components of the TFIID and SAGA transcription complexes (Döring P., unpublished results from our group).

1.5.4 Molecular chaperones as negative regulators

Chaperones such as Hsp90, Hsp70 and their partners can interact with Hsfs to inhibit transcription activation during the normal growth and recovery from the heat stress response.

The physical interactions between Hsp70 and Hsf1 have been shown to occur both *in vitro* and *in vivo* (Abravaya et al., 1992; Baler et al., 1992 and 1996; Nunes and Calderwood, 1995; Rabindran et al., 1994, Westwood et al., 1993). In addition, different studies have revealed that overexpression of Hsp70 in cultured cells either reduces the level of Hsf1 activation during induction (Baler et al., 1996; Mosser et al., 1993) or increases the rate of Hsf1 deactivation (Mosser et al., 1993; Rabindran et al., 1993). More recently, Hsp70 has been shown to repress Hsf1 through direct interaction with the carboxyl-terminal domain (Shi et al., 1998). Thus, the primary autoregulatory role of Hsp70 appears to be downregulation of Hsf1-mediated transcription.

Hsp90 is also a key factor in the regulation of Hsf1 and acts as a repressor. Affinity chromatography and reconstitution experiments *in vitro* (Nadeau et al., 1993; Nair et al., 1996) provided the initial evidence of Hsf1 interaction with Hsp90 and several associated cochaperones. Then several reports have proved *in vitro* and *in vivo* that disruption of the Hsp90 chaperone complex with Hsf1, by means of geldanamycin (Hsp90-binding agent) or microinjection of

individual antibodies against proteins present in the Hsp90 chaperone complex, activates Hsf1, delays recovery, and inhibits Hsf1-mediated transcription after heat stress (Zou et al., 1998; Ali et al., 1998; Bharadwaj et al., 1999). These data suggest a model for Hsf regulation in which Hsp90 forms a complex with Hsf and participates in modulating monomer-to-trimer transitions. In addition multiple components of the Hsp90 chaperone machinery participate in regulating transcriptional activity of Hsf1.

Interestingly, several findings in our group also indicate that Hsp70 and Hsp90 specifically interact with tomato Hsfs (personal data not shown in this work; Kraft M., diploma work, 1999; Schmidt E., diploma work, 1997) and that Hsp90 might have an inhibitory effect on their activity (Scharf K.-D., unpublished results). In addition, the identification of cytoplasmic sHsp(II) as partner of HsfA2 extends further the role of molecular chaperones as regulators of tomato Hsfs activities (see Results and discussion).

1.5.5 Several other Hsfs interacting proteins

Finally, several other factors have been shown to interact with different Hsfs indicating a further regulation of the heat stress response via protein-protein interactions as well as a link between cell stress and other genetic networks. It is noteworthy to mention:

- Heat Shock Factor Binding Protein 1 (HSBP1) interacts with human Hsf1 and has properties of negative regulator of the heat stress response (Satyal et al., 1998);
- *c-myb* proto-oncogene product and p53 tumor suppressor protein interact with chicken Hsf3; they shed light on the molecular events that govern Hsps expression during cellular proliferation and apoptosis (Kanei-Ishii et al., 1997; Tanikawa et al., 2000);
- Phosphatase 2A (PP2A), which provides a mechanism for cross-talk between heat stress response and cell division, interacts with human Hsf2 (Hong and Sarge, 1999);
- Signal Transducer and Activator of Transcription-1 (STAT-1) interacts with human Hsf1; this heteroligomeric complex was shown to produce an

additive effect in activating the *hsp70* and *hsp90β* promoters (Stephanou et al., 1999);

- Nucleoporin p62, a major component of the nuclear pore complex, and Hsf2-BP, a testis-specific protein, interact with the DNA-binding trimer form of human Hsf2 indicating the involvement of an additional regulatory step for transcriptional activation (Yoshima et al., 1997 and 1998).

1.6 The heat stress transcription factors from *Lycopersicon peruvianum*

So far, at least four types of Hsf have been identified in tomato (*Lycopersicon peruvianum*). They have been studied in tomato cell culture, transgenic plants and in heterologous systems like tobacco protoplasts or yeast using reporter assays combining up to 3 Hsfs. The emerging scenario is a network of events which shed light on the complexity of plant heat stress response. The following observations should be noticed in this regard:

- Studies in tomato (*Lycopersicon peruvianum*) cell culture indicate HsfA1 and HsfA3 are constitutive whereas HsfA2 and HsfB1 are heat stress inducible transcription factors. Protein expression levels of HsfA1 and HsfA3 are stable over the time of a heat stress regime whereas it is evident the increasing amount of HsfA2 during the heat stress regime and the rapid decline of HsB1 after the heat stress condition (Fig. 1.6) (Scharf et al., 1998b; Bharti et al., 2000).
- Investigations about their intracellular localization (in tomato cell culture, tobacco protoplasts, *Xenopus oocytes* and CHO-K1 cells) demonstrate that HsfA1 and HsfA3 are distributed between nucleus and cytoplasm under control conditions, but predominantly in the nucleus under heat stress conditions. HsfB1 is found in the nucleus whereas HsfA2 is in the cytoplasm. HsfA2 needs the presence of HsfA1 for retention in the nucleus under ongoing heat stress. Moreover, HsfA2 is the only tomato Hsf which localizes in the heat stress granules (HSGs), large cytoplasmic aggregates (approx. 40 nm in size) formed during prolonged and severe heat stress conditions (Scharf et al., 1998b; Bharti et al., 2000, Heerklotz et al., 2001).

- In transient tobacco protoplasts assays the coexpression of two or more different Hsfs leads to an increase in reporter activity. A synergistic interaction is observed between tomato HsfA1 and HsfB1 (unpublished results from our group).
- Little is known about their developmental regulation. In addition, analyses of transgenic plants (*Lycopersicon esculentum* cv Moneymaker by *Agrobacterium*-mediated gene transfer) expressing increased or decreased levels of tomato Hsfs suggest that tomato Hsfs may not have a direct role in plant development, at least at normal temperature conditions (Mishra S.-K., diploma work, 2000).

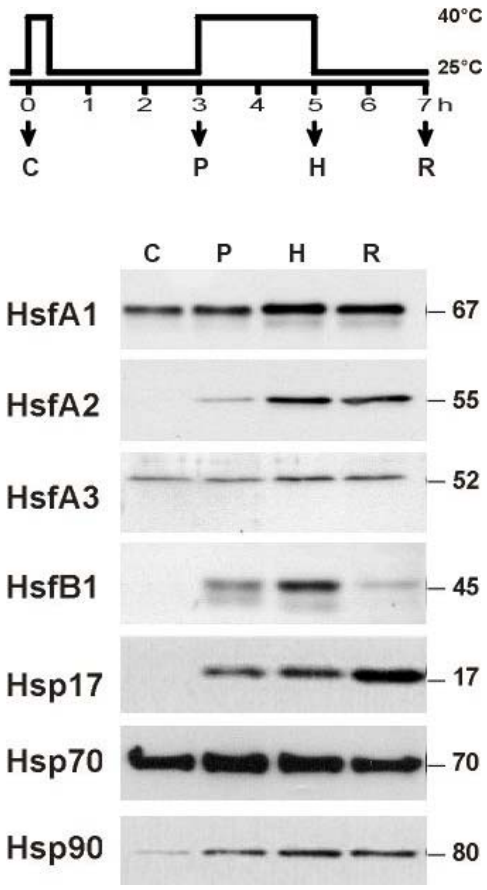


Figure 1.6 Expression of Hsfs and Hsps in tomato (*Lycopersicon peruvianum*) cell culture under different conditions. Protein extracts (10 μg) were prepared from tomato cell culture under control (C), preinduced (P), heat stress (H) and recovery (R) conditions. The heat stress treatment was as indicated in the pictograph at the top of the panel. Samples were analyzed by SDS-PAGE and Western blotting as described in Materials and methods. Molecular masses (in kDa) are indicated on the right.

1.7 Purpose of this study

In tomato cell culture, the simultaneous expression of four Hsfs under heat stress conditions results in a system with dynamically changing cellular levels, intracellular localization and functional interactions, which is far too complex to investigate the role of single Hsfs. As already mentioned, protein-protein interactions play an important role in regulating Hsfs activity and have also revealed their functions in novel genetic regulatory pathways. Therefore our aim was to investigate the protein interactions *in vitro* and *in vivo* involving all tomato Hsfs as an approach to understand the relevance of Hsf multiplicity and to get more insights into the complexity of the plant heat stress response. The following aspects were considered:

- interactions between Hsfs themselves;
- interactions between Hsfs, in particular tomato HsfA2, and small heat stress proteins;
- identification of novel Hsf-interacting proteins by pull-down assay.

As part of this study, the two-hybrid and the pull-down assays were also used to evaluate whether these two complementary techniques could provide the same results.

2 Materials and methods

2.1 General materials and methods

2.1.1 Recombinant DNA manipulations

Standard procedures were used for plasmid preparation, gene cloning and nucleic acid analysis (Sambrook et al., 1989; Ausubel et al., 1993). Plasmids were maintained and propagated in *Escherichia coli* strain DH5α (Hanahan, 1983). Deletions and mutations were generated by PCR using site-specific primers or using already existing enzymatic restriction sites. Accuracy of the constructs was confirmed by sequencing. Plasmid maps and other relevant details regarding the vectors used in this study are reported in Appendix C. A complete listing of the oligonucleotides used to create point mutations, linker mutations and to sequence the new generated plasmids is reported in Appendix B.

2.1.2 Enzymes

All restriction endonuclease enzymes were supplied by Roche Diagnostic or Fermentas MBI, unless otherwise indicated.

2.1.3 DNA sequencing

For DNA sequencing plasmid purification was performed by using QIAprep Spin Miniprep Kit (QUIAGEN). A DNA sequencing service was provided by several companies (BioSpring GmbH, Frankfurt; Sequence Laboratories, Göttingen GmbH; MWG-BIOTECH, Ebersberg).

2.1.4 Polymerase chain reaction (PCR)

PCR was performed according to Innis et al. (1990). PCR products were amplified using the Taq^{PLUS}-Precision-PCR-Sysem (STRATAGENE) and a PE9600 thermal cycler (Perkin-Elmer, Applied Biosystem, Langen). Standard PCR conditions were:

Condition	Temperature	Time	
Denaturation:	94°C	5 min	
Denaturation:	94°C	30 s	30 cylces
Annealing:	55°C	30 s	
Polymerization:	72°C	1 min	

2.1.5 Buffers and solutions

Unless otherwise indicated, buffers and other solutions were prepared according to Sambrook et al. (1989) and Ausubel et al. (1993).

2.1.6 Protease inhibitors

- Protease inhibitors were purchased from SIGMA:

Name	Working solution
Aprotinin	2 μg/ml
Leupeptin	1 μg/ml
Pefabloc	10 μg/ml
Pepstatin A	1 μg/ml
TLCK	1 μg/ml
TPCK	2 μg/ml

- Protease inhibitors tablets (Boehringer Mannheim) were dissolved according to manufacturer's instructions.

2.2 Yeast strains and growth conditions

The haploid *Saccharomyces cerevisiae* RSY4 strain, derivative of the RSY10 (*MATa/MAT*α, *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*) strain, was the recipient for all Hsf substitutions. Yeast 2µ plasmid (pAD4Δ or pAD5Δ; Ballester et al., 1989) with auxotrophy marker leucine (LEU2) and alcohol dehydrogenase (*ADH1*) gene promoter and terminator was used for expressing different Hsfs (see Appendix C). The FOA-selection procedure for functional replacement of the yeast Hsf by tomato Hsfs was performed as previously described by Boscheinen et al. (1997). Yeast was transformed and grown according to standard protocols (Rose et al., 1990).

2.2.1 Preparation of yeast cell extracts

Liquid cultures were daily grown at 28°C up to 0.6-1 OD_{600} and then processed for protein extract preparation.

2.2.1.1 Denatured protein extract of yeast cells

- 2 OD_{600} units of cells were transferred to a tube containing 2 ml of 50 mM Tris-HCl pH 7.5 and 10 mM NaN_3 on ice.
- Cells were harvested at 5000 rpm, for 5 min at 4°C and resuspended in 30 µl of EBS buffer (80 mM Tris-HCl pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 1.5% DTT (w/v) and 0.1 mg/ml Bromophenol blue).
- Samples were heated at 95°C for 3 min to rapidly inactivate proteases and approx. 0.1 g of 0.2-mm glass beads (Sigma) were added until they reached the top of the liquid.
- After vigorous mixing for 2 min and addition of 70 µl of EBS buffer, samples were mixed and heated at 95°C for 5 min (or 37°C for 10 min for very hydrophobic proteins).
- 5-20 µl of the protein extract was analyzed by SDS-PAGE.

2.2.1.2 Whole cell extract of yeast cells

- Cells were harvested at 5000 rpm, for 15 min at 4°C and washed with ice-cold sterile water. Then, cells (0.5 mg/ml, wet w/v) were resuspended in H-buffer (25 mM HEPES-KOH pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol (v/v) and 1% β-mercaptoethanol) with protease inhibitors and lysed by sonication (Branson Sonifier B-12, microtip, 70 W) for 1 min, three times.
- After addition of NaCl to a final concentration of 500 mM and Nonidet-P40 to a final concentration of 0.2% (v/v), the homogenate was kept on ice for 20 min, centrifuged at 15000 rpm, for 15 min at 4°C.
- The supernatant (whole cell extract) was frozen in liquid nitrogen and stored at -90°C.

2.3 Tomato cell culture and growth conditions

The origin, growth characteristics and nutrient medium of the suspension cell culture of *Lycopersicon peruvianum Lp VII* have been described by Nover et al. (1982). Propagation and incubation of control cultures were performed at 25°C on rotary shakers at 135 rpm in a modified MS medium (Murashige and Skoog, 1962). Exponentially growing cell cultures with an optical density (A_{578}) of about 0.9 were used in the experiments.

The following heat-stress regime was applied: 15 min at 40°C (pulse of heat stress condition), 3 h at 25°C (pre-induced condition), 2 h at 40°C (heat stress condition), and 2 h at 25°C (recovery condition) (see pictograph in Fig. 1.6). In parallel, part of the culture was grown at 25°C for the same total time (control culture). Cells were harvested by filtration, quickly washed, weight (wet weight) and frozen in liquid nitrogen.

2.3.1 Whole cell extract of tomato cells

- Cells were thawed on ice, resuspended (2 mg/ml, wet w/v) in NEB500 buffer (25 mM HEPES-KOH pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 10% glycerol (v/v), 0.2% Nonidet-P40 (w/v) and 10 mM β-mercaptoethanol) with protease inhibitors and lysed by sonication (Branson Sonifier B-12, microtip, 70 W) for 1 min.
- Samples were centrifuged at 10000 rpm, for 30 min, at 4°C and the supernatant (whole cell extract) was frozen in liquid nitrogen and stored at -90°C.
- The quality of each preparation was assessed by analyzing the heat stress inducible Hsfs (e.g. HsfA2) by Western blot analysis (2.7.2).

2.4 Expression and purification of recombinant tagged-proteins

The cDNA of HsfA1, HsfA3 and HsfB1 were inserted into the bacterial expression vector pET or pCJ20 in frame with a tag of 6 histidine (His_6-tag) at the N- or C-terminus. The cDNA of Lp-Hsp17.6(I), Lp-Hsp17(II), Lp-Hsp17(IIs), Ps-Hsp17.7(II) and At-Hsp17.6(II) were inserted into the bacterial expression vector pGEX carrying the Glutathione S-transferase sequence (GST-tag) at the N-terminus (Appendix C). These vectors were used to transform the *E. coli* BL21(DE3) or BL21(DE3)(pLysS) strain (Studier et al., 1987). Protein expression was induced at an A_{600} of 0.2-0.4 OD by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM for 2 h at 37°C.

- Expression of recombinant proteins was verified by separating pre- and post-IPTG induced samples on 12% SDS-polyacrylamide gels, followed by Coomassie staining.
- Post-IPTG induced cells were harvested by centrifugation at 5000 rpm, for 20 min, at 4°C, washed with sterile water and resuspended in extraction buffer (see paragraphs below) at 2-5 ml per gram wet weight.
- After addition of lysozyme (1 mg/ml) and incubation on ice for 30 min, the sample was lysed by sonication (Branson Sonifier B-12, 200 W, 30% duty cycle) for 3 min, three times.

- The homogenate was centrifuged at 15000 rpm, for 30 min, at 4°C to pellet the cellular debris. Then, the supernatant was transferred to a fresh tube and incubated with Ni-NTA Sepharose CL-6B (Quiagen) to purify His_6-tagged protein or with Gluthatione Sepharose 4B (Pharmacia) to purify GST-tagged protein according to manufacturer's instructions.

Purification was carried out by batch procedure, gently rotating the recombinant tagged-protein and the affinity-resin at 4°C for at least 1 h. Washing steps (6 times) were performed by adding ice-cold washing buffer (10 times the sample volume) and further gently mixing at 4°C. The sample was recovered by centrifugation at 500 rpm, at 4°C for 10 min. In the case of His-tagged proteins, elution was performed once or twice by gently mixing the sample at 4°C for 30 min in elution buffer.

2.4.1 Purification under native conditions for His_6-tagged soluble proteins

HsfA1 and HsfB1 recombinant proteins were extracted and purified as previously described by Boscheinen (1997, PhD thesis) with some changes as reported below:

- Cells were resuspended in extraction buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5% glycerol (v/v), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and protease inhibitors); then samples were adjusted to 0.2% Nonidet-P40 (v/v) and 500 mM NaCl.
- Washing steps were carried out in extraction buffer with addition of imidazole at a final concentration of 30 mM for N-terminal His_6-tagged HsfA1 and HsfB1, or 50 mM imidazole for C-terminal His_6-tagged HsfA1 and HsfB1 proteins.
- Elution was achieved by adding extraction buffer with addition of imidazole at the final concentration of 100 mM for N-terminal His_6-tagged HsfA1 and HsfB1, 250 mM imidazole for C-terminal His_6-tagged HsfA1, and 200 mM for C-terminal His_6-tagged HsfB1, HsfB1 CTD and HfB1 DBD proteins.

- Purified proteins were dialyzed in 500-buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% glycerol (v/v), 2.5 mM $MgCl_2$, 1 mM β-mercaptoethanol and 0.5 mM EDTA) at 4°C for 3 h, and then in 200-buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5% glycerol (v/v), 2.5 mM $MgCl_2$, 1 mM β-mercaptoethanol and 0.5 mM EDTA) at 4°C for 3 to 12 h.

2.4.2 Purification under native conditions for GST-tagged soluble proteins

Lp-Hsp17.6(I), Lp-Hsp17(II), Lp-Hsp17(IIs) and At-Hsp17.6(II) recombinant proteins were extracted and purified according to manufacturer's instructions (Pharmacia) with some changes as reported below:

- Cells were resuspended in extraction buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 1mM EDTA, 1 mM DTT and protease inhibitors); then the sample was adjusted to 0.2% Nonidet-P40 (v/v) and 500 mM NaCl.
- Glutathione-resin suspension was saturated in 0.5% (w/v) BSA (fraction V, SIGMA) before use.
- Washing steps were performed twice in extraction buffer with 500 mM NaCl, twice in extraction buffer with 200 mM NaCl and then twice in extraction buffer with 150 mM NaCl.
- GST fusion proteins newly bound to gluthatione-resin were directly used for the pull-down assays (2.5.2.3).

2.4.3 Purification under native conditions for insoluble proteins

For His_6-HsfA3 and GST Ps-Hsp17.7(II) recombinant proteins, the extraction procedure was an adaptation of an approach previously described by Frangioni and Neal (1993).

- Cells were resuspended in STE buffer (10 mM Tris-HCl pH 8, 150 mM NaCl and 1mM EDTA) at 2-5 ml per gram wet weight.
- After addition of lysozyme (1 mg/ml), the sample was incubated on ice for 15 min.

- The sample was lysed by sonication (power level 4.5% duty cycle) in a water bath after addition of DTT to a final concentration of 5 mM, protease inhibitors and N-laurylsarcosine (Sarkosyl, Sigma) to a final concentration of 1.5% (v/v).
- The homogenate was centrifuged at 15000 rpm, for 30 min, at 4°C to sediment the cellular debris.
- The supernatant was transferred to a fresh tube and Triton X-100 was added to a final concentration of 2% (v/v).
- The lysate was incubated with affinity-resin suspension to purify His_6-HsfA3 and GST Ps-Hsp17.7(II) as described in paragraphs 2.4.1 and 2.4.2 respectively.

2.5 Protein-protein interaction assays

2.5.1 Cross-linking assay

- Yeast whole cell extract was diluted in H-buffer (2.2.1.2) to approximately 0.5 mg/ml total protein in 150 mM NaCl final concentration. Aliquots of 200 μl were incubated for 30 min at room temperature before adding ethyleneglycol-bis (sucinic acid N-hydroxysuccinimide) ester (EGS; Sigma) freshly diluted from a 200 mM stock solution in DMSO. The reaction was quenched after 30 min by the addition of 30 mM lysine. Proteins were precipitated with trichloroacetic acid (6% final concentration), washed twice with 70% ethanol containing 20 mM Tris base and finally dissolved in SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 2.5% β-mercaptoethanol (v/v) and 0.1% bromophenol blue (w/v)).
- The cross-linked products were separated on 6% or 8% SDS-PAGE gels.
- Western blot analysis was performed by using a specific antibody for the cross-linked Hsf.

2.5.2 Pull-down assays

The pull-down assay is an appropriate method to identify and to characterize the physical interaction between proteins *in vitro*. The advantages of this protein affinity chromatography technique are its sensitivity and the ability to test all proteins in an extract equally. Moreover it can detect interactions dependent on a multisubunit tethered protein, unlike the case with the two-hybrid assay. The fundamental principles are outlined in the flowchart below (Fig. 2.1).

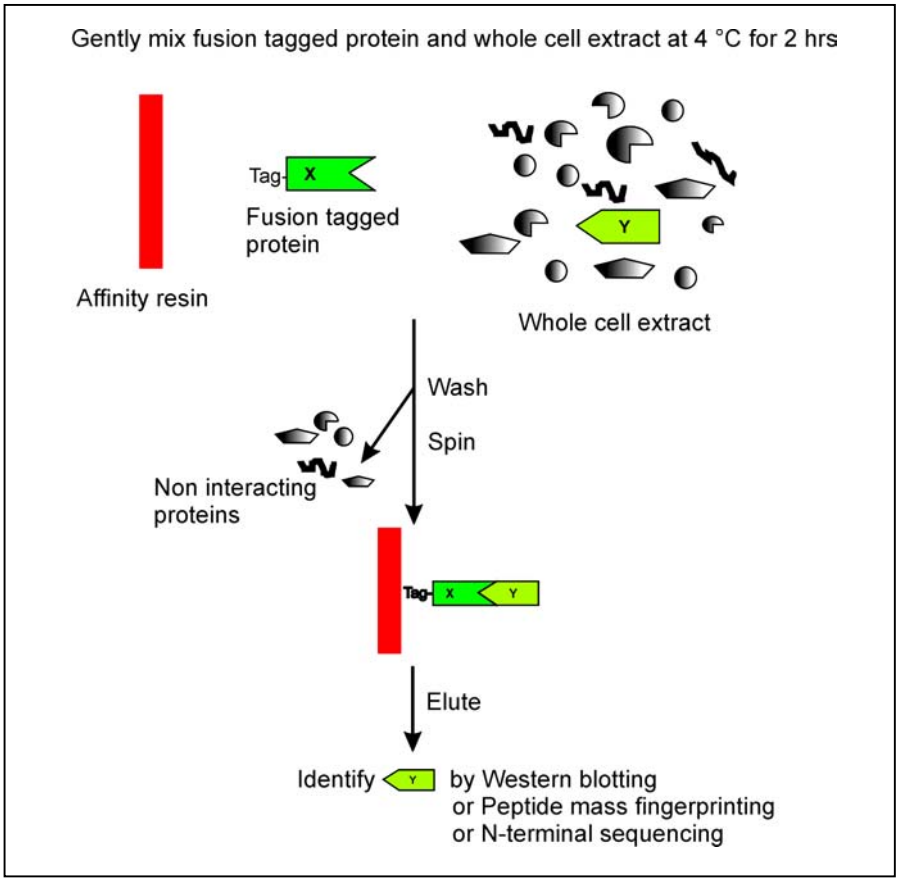


Figure 2.1 Flow diagram: pull-down assay. A fusion tagged-protein (**X**: His- or GST-tagged protein) is coupled to an affinity-resin (Ni-NTA or glutathione resin) via the tag and used to select a protein (**Y**) that is retained from an appropriate extract (yeast or tomato whole cell extract). During the washing steps non-interacting proteins are washed off and then the retained protein can be eluted and identified. Identification can be achieved by Western blotting or by other techniques as Peptide mass finger printing or N-terminal sequencing.

2.5.2.1 Pull-down assay with yeast whole cell extract using His-tagged proteins

Recombinant and purified N-terminal His_6-tagged Hsf proteins (0.1, 0.3 and 3.6 μg) were mixed with different and increasing concentrations of yeast whole cell extracts (50, 150 and 450 μg) from a strain expressing different tomato Hsfs. The whole cell extract from the RSY4 strain was added to each reaction mixture to bring the total amount of proteins to 10 μg/μl in 500 μl reaction volume of interacting buffer (10 mM HEPES-KOH pH 7.4, 175 mM NaCl, 5 mM $MgCl_2$, 5% glycerol (v/v), 10 mM NaF, 1 mM β-mercaptoethanol, 0.1% Nonidet-P40 and 10 mM imidazole) in the presence of protease inhibitors. Samples were incubated and gently mixed for 2 h. Then, 15 μl of Ni-NTA resin suspension were added and the samples were rocked for 30 min, washed three times for 10 min with 1 ml of washing buffer (interacting buffer with 30 mM imidazole). Bound proteins were eluted by boiling samples in 30 μl SDS-elution buffer (2xSDS-sample buffer diluted (1:1) in interacting buffer). Half of each sample was analyzed by 12% SDS-PAGE gel and Western blotting.

2.5.2.2 Pull-down assay with tomato whole cell extract using His-tagged proteins

Recombinant and purified C-terminal His_6-tagged Hsf proteins were used to saturate a Ni-NTA resin in order to create Hsf-resins. Then, 25 μl of Hsf-resin were incubated with tomato whole cell extract (500 μg) in a final volume of 500 μl of interacting buffer (25 mM HEPES-KOH pH 7.4, 175 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 5 % glycerol (v/v), 0.1% Nonidet-P40 (v/v), 10 mM imidazole) for 2 h. Samples were washed three times in 1 ml interacting-200-buffer (interacting buffer but 200 mM NaCl as final concentration) and then other three times in 1 ml interacting-500-buffer (interacting buffer but 500 mM NaCl as final concentration). Each washing step was for 10 min. Elution was performed by adding stepwise high-salt-elution buffer (interacting buffer but 1 M NaCl as final concentration), imidazole-elution buffer (interacting buffer but 0.3 M imidazole as final concentration) and SDS-elution buffer (2xSDS-sample

buffer diluted (1:1) in interacting buffer). During each elution step samples were gently mixed for 15 min. All procedures were performed at 4°C and all buffers contained protease inhibitors. Samples were analyzed by 12% or 15% SDS-PAGE gel and silver-staining. Protein identification was achieved by Peptide mass finger printing (2.7.3) and N-terminal sequencing (2.7.4) analyses.

2.5.2.3 Pull-down assay with yeast whole cell extract using GST-tagged proteins

Recombinant proteins (5 μg) bound to 15 μl glutathione-agarose suspension were equilibrated in interacting buffer (10 mM HEPES-KOH pH 7.4, 175 mM NaCl, 5 % glycerol (v/v), 0.1 % Nonidet-P40 (v/v), 0.1 % BSA (w/v), 5 mM $MgCl_2$, 1 mM EDTA and 10 mM NaF). Then, the protein affinity-resins were incubated with yeast whole cell extract (50, 150 and 450 μg) in 500 μl reaction volume of interacting buffer for 2 h at 4°C. Samples were washed three times using 1 ml of washing buffer (10 mM HEPES-KOH pH 7.4, 175 mM NaCl, 5 % glycerol (v/v), 0.2 % Nonidet-P40 (v/v), 0 5 mM $MgCl_2$, 1 mM EDTA and 10 mM NaF) in the presence of 0.1 % BSA (w/v) and three times more without BSA. Bound proteins were eluted in 30 μl SDS-elution buffer (2xSDS-sample buffer diluted (1:1) in interacting buffer) by heating at 95°C for 5 min. Eluted proteins were analyzed by 12% SDS-PAGE gel and Western blot analysis.

2.5.3 Yeast two-hybrid assay

The two-hybrid assay, developed by Fields and Song (1989), is a yeast-based assay that uses transcriptional activity as a measure of protein-protein interaction *in vivo*. It relies on the modular nature of many site-specific transcriptional activators, which consists of a DNA-binding domain and a transcriptional activation domain. The DNA-binding domain serves to target the activator to the specific genes that will be expressed and the activation domain contacts other proteins of the transcriptional machinery to enable transcription to occur. General features are outlined in Figure 2.2.

For the direct testing of protein-protein interactions, the two-hybrid expression vectors (Stratagene), pBD Gal4, which has tryptophan as selectable marker and encodes the yeast Gal4 DNA-binding domain (amino acid residue 1 to 147) and pAD Gal4, which has leucine as selectable marker and encodes the

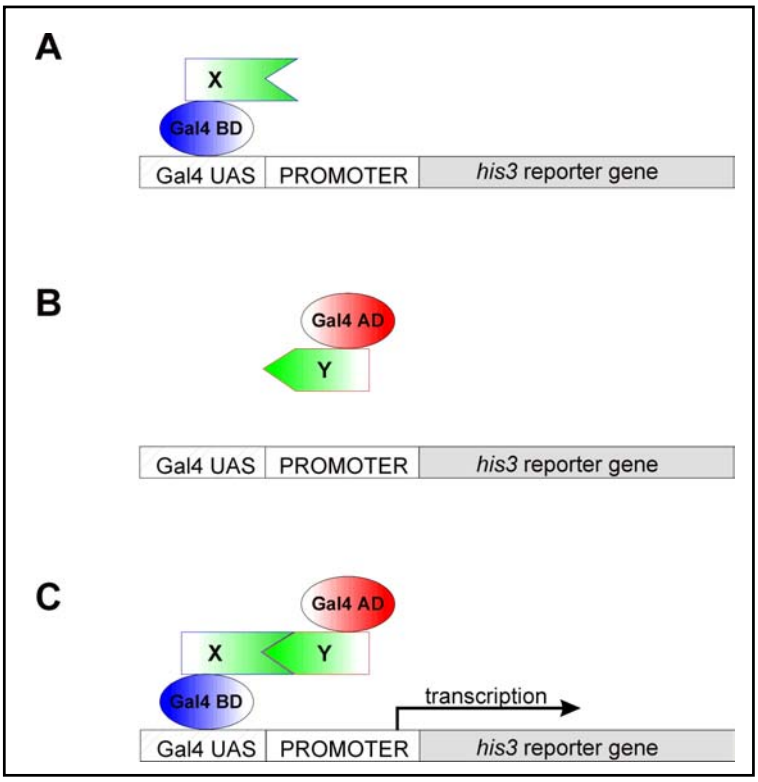


Figure 2.2 Basic features of the yeast two-hybrid system. (**A**) A fusion protein of Gal4 DNA-binding domain (Gal4 BD) and protein **X** binds to the Gal4 UAS (upstream activating sequence) but can not activate transcription if protein **X** does not contain an activation domain. (**B**) A fusion protein of Gal4 activation domain (Gal4 AD) and protein **Y** does not activate transcription because it does not localize to the UAS. (**C**) Interaction between **X** and **Y** is necessary to reconstitute Gal4 function and results in transcription of an appropriate reporter gene (e.g. *his3*).

yeast Gal4 activation domain (amino acid residue 768 to 881), were used for the construction and expression of hybrid fusion proteins, baits and preys respectively (see Appendix C).

A standard protocol from CLONTECH laboratories was used for the assay (schematic description of the procedure is reported in Fig. 2.3).

- Plasmids encoding the two hybrid proteins, bait (pBD Gal4 fusion constructs) and prey (pAD Gal4 fusion constructs) were cotransformed into *S. cerevisiae* host strain YRG-2 (genotype: MATα, *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3 112, *gal*4-542, *gal*80-538, LYS::UAS$_{GAL1}$-TATA $_{GAL1}$-HIS3, URA3::UAS$_{GAL4\ 17mers(x3)}$-TATA $_{CYC1}$-*LacZ*; reporter: *lacZ*, *his3*; transformation markers: leu2, trp1) provided from Stratagene.
- Cotransformants were initially selected on solid synthetic minimal media lacking tryptophan and leucine.
- Interactions between bait and prey were assessed qualitatively by growth of cotransformants on solid synthetic selective media lacking tryptophan, leucine and histidine. Cotransformation of bait and prey vectors in the yeast YRG-2 strain reconstitute a transcription factor able to activate transcription of the *his3* reporter gene under the control of a *gal4* promoter. Thus under these circumstances and under conditions of histidine starvation, survival of yeast (growth) can be directly correlated to the extent of which the *his3* promoter is activated, providing a direct assessment of protein interaction.
- Recombinant hybrid proteins were tested for no self-activation and nonspecific protein-binding by cotransforming preys with the pBD Gal4 vector and baits with the pAD Gal4 vector.
- 3-amino-1,2,4-triazole (3-AT, Sigma), competitive inhibitor of the imidazolyl glycerol phosphate dehydratase encoded by the *his3* gene, was added to the selective media at the concentration of 5 mM to suppress basal level of HIS3 expression induced by the baits, i.e. HsfA2 and HsfA3 baits.
- The assay was always performed using three individual colonies for each cotransformation; every time a positive (HsfA2 homodimeric interaction) and a negative (empty pBD Gal4 and pAD Gal4 vectors) controls were included.

In this study the growth behaviour of only one yeast strain streak is shown for each cotransformation; pictures were taken from colonies grown after 3 days at 30°C.

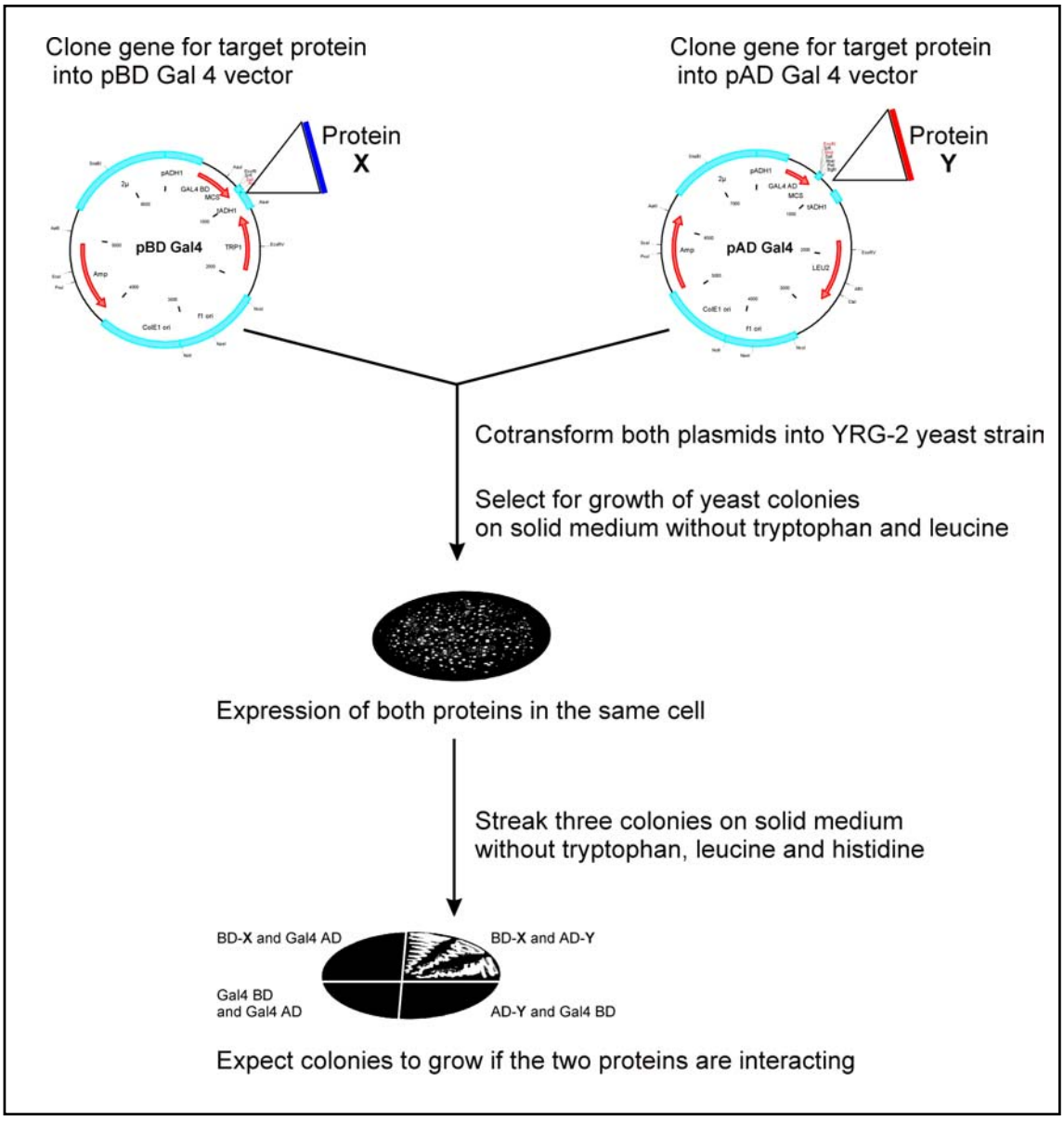


Figure 2.3 Flow diagram: Two-hybrid assay for testing an interaction between known proteins

2.6 Sample preparation and gel-electrophoresis

Protein concentrations were determined by using the Bio-Rad Protein-Assay-regent (BioRad Laboratories, München). Bovine serum albumine (BSA fraction V, SIGMA) served as standard. Proteins were analyzed by gel-electrophoresis. Samples were mixed with an equal volume of 2xSDS-sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 0.2% bromophenol blue (w/v) and 20% β-mercaptoethanol (v/v) or 200 mM DTT), heated for 5 min at 95°C and separated on continuous SDS-PAGE at constant voltage (50-80 V) at room temperature (Sambroock, 1989). After electrophoresis, gels were either silver- or Coomassie-stained; alternatively gels were transferred to nitrocellulose or PVDF membranes for Western blot analysis or N-terminal sequencing analysis (see 2.7).

Standard protein molecular weight markers were purchased from Pharmacia: thyroglobulin (330 kDa), half unit of ferritin (220 kDa), phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 or 46 kDa), carbonic-anhydrase (30 kDa), trypsin-inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.7 Protein detection and identification methods

2.7.1 Staining procedures

Standard methods were used for Coomassie and Ponceau stainings (Sambrook et al., 1989; Ausubel et al., 1993). The silver staining procedure was as reported below.

- Gels were soaked in the fixing-solution (33% (v/v) ethanol, 11% (w/v) acetic acid) for 30 min.
- Then, the solution was poured out and the incubation-solution (30% (v/v) ethanol, 0.8 M CH_3COONa, 0.125% (w/v) glutaraldehyde, 8 mM $Na_2S_2O_3x5H_2O$) was added for 30 min.

- After rinsing gels in deionized water for three times, they were soaked in the staining-solution (1.8 mM $AgNO_3$, 0.0074% (w/v) formaldehyde) for 20 min.
- Gels were placed into clean containers and incubated in the developing-solution (0.2 M Na_2CO_3, 0.0037% (w/v) formaldehyde) for 1-10 min and then in 2% (w/v) acetic acid for 5-10 min.
- Finally gels were rinsed in deionized water for at least 1 h.

2.7.2 Western blot analysis

Proteins were blotted onto nitrocellulose membrane (Schleicher and Schuell) at constant voltage (2 V/cm) using A- and K-transfer buffers (Roth) for 1 h at room temperature. Then the membrane was coated with a blocking reagent (5% (w/v) solution of milk in PBS buffer) for 1 h at room temperature (or overnight at 4°C). Western analysis was performed using antibodies specific to tomato Hsfs (Lyck et al., 1997) and sHsp (Forreiter et al., 1997) for 2 h at room temperature (see Table 2.1). The second antibody was a horseradish-peroxidase-conjugated rabbit anti-goat IgG (Sigma), used at a dilution of 1: 10000 in 5% (w/v) solution of milk in PBS buffer, for 1 h at room temperature. Protein detection was achieved using the ECL detection kit (NEN, Life science products, U.S.A.) as indicated by the manufacturer's protocol.

First antibody	Dilution	Antigen used to raise the antibody
Anti HsfA1	1: 5000	Recombinant His_6-tag Lp-HsfA1 (aa 16-527)
Anti HsfA2	1: 5000	Recombinant His_6-tag Lp-HsfA2 (aa 1-351)
Anti HsfA2(pep6)	1: 5000	Peptide of last 15 amino acids at the C-terminus of Lp-HsfA2
Anti HsfB1	1: 5000	Recombinant His_6-tag Lp-HsfB1 (aa 1-293)
Anti sHsp cl I	1: 5000	Recombinant His_6-tag Ps-Hsp 18.1(I) (aa 7-143)
Anti sHsp cl II	1: 2500	Recombinant His_6-tag Ps-Hsp 17.7(II) (aa 2-157)

Table 2.1 First antibodies. Description and technical details for the used first antibodies.

2.7.3 Peptide mass fingerprinting analysis

This technique involves generation of peptides from proteins using residue-specific enzymes (i), determination of peptide characteristics by mass spectrometry (MS) (ii), and matching of these masses against theoretical peptide libraries generated from protein sequences databases (iii). The identification can be achieved when lists of best-matching proteins are compared for identical database entries. All these analyses were performed in collaboration with C. Scharf (Department of Microbiology, University of Greifswald) and procedures were as reported below:

i. Sample preparation

- **Tryptic digestion of protein within acrylamide gel pieces**

- The SDS-PAGE was Coomassie-stained for 15-30 min and then it was destained for 30-60 min until protein bands were visible.
- Each desired band (as a gel piece) was cut out, placed in a 1.5 ml microcentrifuge tube and covered with 0.5 ml of trypsin-digestion solution 1 (100 mM Tris-HCl pH 8.5 and 50% (v/v) acetonitrile). Samples were incubated at 30°C for 20 min.
- After addition of 0.5 ml of trypsin-digestion solution 2 (100 mM Tris-HCl pH 8.1 and 10% (v/v) acetonitrile) samples were again incubated at 30°C for 20 min.
- Supernatants were discarded and samples were dried in a speedvac for 20-30 min.

- **Enzymatic digestion**

- Samples were incubated in 30 μl of trypsin-digestion solution 3 (1 μg (w/v) trypsin (Promega), 100 mM Tris-HCl pH 8.1, 10% (v/v) acetonitrile and 1 mM $CaCl_2$) at 37°C overnight with gentle shaking.

- **Protein extraction**

- Samples were incubated at room temperature with gently shaking overnight after addition of 100 μl of stop-elution solution (75% (v/v) acetonitrile and 2% (v/v) trifluoroacetic acid).
- Each supernatant was transferred to a clean microcentrifuge tube and concentrated in a speedvac.
- After addition of 10 μl of loading-solution (30% (v/v) acetonitrile and 1% (v/v) trifluoroacetic acid), samples were incubated at room temperature for 15 min.

ii. MALDI-TOF analysis

This technique requires the sample to be crystallized with a vast excess of an appropriate chemical matrix (e.g. α-cyano-4-hydroxycinnamic acid) on a specially designed target (sample-plate), then, the combined solid mixture is inserted into the ionization source of the matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems Voyager-DE^{TM} STR, Applied Biosystems) where it is bombarded and ionized by a laser. The matrix transforms the laser energy into excitation energy for the sample and also enhances sample ion formation by participating in the proton transfer mechanism. In the analyzer the ions are separated according to their mass-to-charge (m/z) ratios and in the detector the signal from the separated ions is detected, amplified and sent to a data system for processing. The obtained MS spectrum gives information on the molecular weight for each peptide derived from the sample (Fig. 2.4).

- **Protein preparation for MALDI-TOF measurements**

- An aliquot (0.5-2 μl) of the peptide solution was diluted with an equal volume either of the peptide-sample solution (30 mg/ml α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile) or of the protein-calibration solution (protein calibration mix shown in Table 2.2, 30 mg/ml α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile).

calibration compounds	monoisomer
Angiotensin I:	1296.6853
ACTH1-17 :	2093.0867
ACTH1-39 :	3657.92294

Table 2.2 Protein calibration mix (Sigma)

- Each aliquot (1-2 μl) of the final solution was applied to the MS-target, dried at room temperature for several min and analyzed by MALDI-TOF.

iii. Amino Acid composition identification

Once a molecular weight has been determined for each peptide in the sample, amino acid sequence for each peptide can be deduced at least partially by means of software programs (PepIdent and MS-Fit).

Bioinformatic facilities used:
http://www.expasy.ch/tools/pepident.html; database: SWISS-PROT
http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm; database: NCBInr-Database

2.7.4 Edman degradation 'sequence tag' identification

Samples were resolved on SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane (DuPont-NEN) and dried. PVDF-bound proteins were eluted and sequenced using the automated sequencer (Applied Biosystems) by C. Scharf (Department of Microbiology, University of Greifswald). Modern automated protein sequencers utilize the Edman degradation method originally developed in the 1950 by Pehr Edman. Sequencing was performed for 5 cycles to create an N-terminal 'sequence tag' and identity was established by matching the sequence obtained against those in protein databases (SWISS-PROT database).

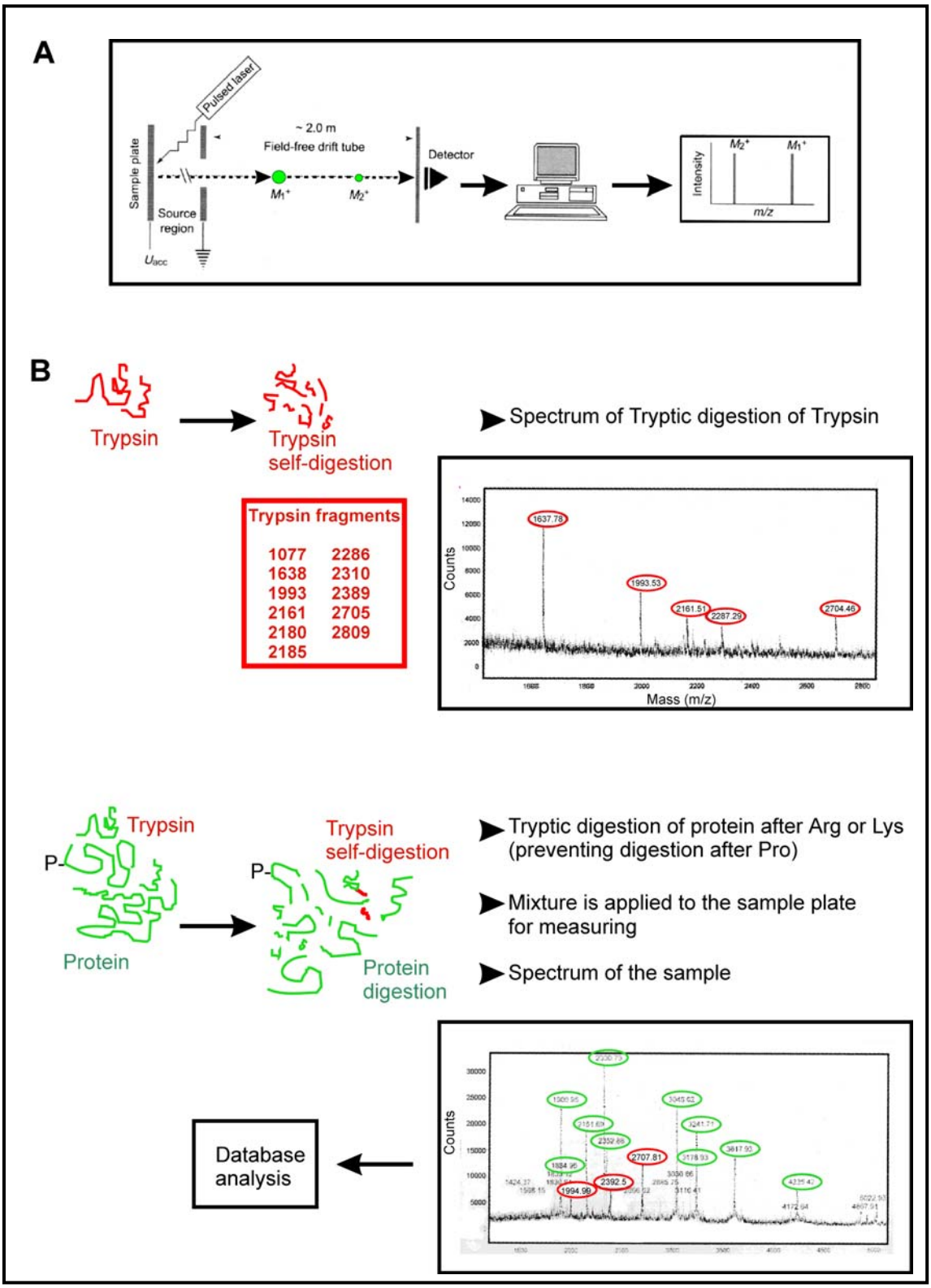


Figure 2.4 A schematic representation of a MALDI-TOF mass spectrometry analysis. (**A**) Matrix and analyte ions are desorbed and ionized upon irradiance with a laser pulse in the source region; a potential (U_{acc}) applied to the sample plate accelerates the ions into the field-free drift tube. Ions with smaller mass-to-charge ratios (m/z) travel down the drift tube at a higher velocity than ions with a larger m/z. The time-of-flight of each ion is measured and, with proper calibration, converted into a corresponding m/z. This diagram shows the separation and detection of two positive, single-charged ions with different masses, M_1 and M_2. (**B**) Flow diagram: Spectrum of a sample analysis by MALDI-TOF mass spectrometry analysis. A spectrum is a mixture of picks derived from the protein fragments after trypsin cleavage and from fragments of the self-cleavage of trypsin.

2.8 Transient reporter assay in tobacco protoplasts

Preparation of mesophyll protoplasts from *Nicotiana plumbaginifolia* plants, polyethylene glycol-mediated transformation and GUS assay were performed as previously described by Treuter et al. (1993). The Hsf-dependent *phsp17** reporter plasmid (Schöffl et al., 1989) contains the promoter region (base pairs –321 to -12) of the soybean *hsp17.3B* gene fused to the minimal 35S cauliflower mosaic virus (*CaMV*) promoter upstream of the *gus* gene (Treuter et al., 1993). $3x10^4$ tobacco protoplasts were transformed with 2 μg of *phsp17** reporter and 0.5 μg of each class A Hsf (or 0.75 μg of HsfB1) expression plasmids. The total amount of DNA was 4 μg; this concentration was kept constant by addition of a plasmid DNA consisting of the neomycin phosphotransferase gene under the control of the full-length *35 CaMV* promoter (pRT103/neo) (Treuter et al., 1993).

The measurement of β-glucuronidase (GUS) reporter activity was based on the method described by Jefferson (1987) with several modifications:

- Protoplasts were harvested by centrifuging at 15000 rpm for 5 min at 4°C.
- Samples were resuspended in 50 μl of GUS extraction buffer (Jefferson, 1987) and immediately frozen in liquid nitrogen.
- Samples were thawed and each 25 μl of the lysate mixed with 25 μl of 1 mM MUG solution (4-methyl-umbelliferyl-β-D-glucuronide in GUS extraction buffer) were incubated for 1 to 3 h at 37°C in a microtiter plate for measurement of GUS activity.
- Fluorescence was measured in a FLUOstar microtiter plate reader (BMG LabTechnologies GmbH, Offenburg, Germany). All GUS values result from the mean of at least three independent transformations.

3 Results and Discussions

3.1 Protein interactions between members of the Hsf family

Previous data from our group (Scharf et al., 1998b; Lyck et al., 1997; unpublished data) have indicated that a network of different protein interactions may have significant influence on the function and intracellular localization of tomato Hsfs during the heat stress response:

- HsfA2 and HsfA3 were identified in a yeast two-hybrid screening using HsfA1 as bait and a cDNA library from heat stress tomato cell culture.
- HsfA2 needs interaction with HsfA1 for nuclear localization. HsfA2 homotrimer localizes in the cytoplasm due to an efficient nuclear export. Formation of the HsfA2/HsfA1 heterooligomers was confirmed by coimmunoprecipitation.
- In the tobacco protoplasts reporter assay the coexpression of HsfA2 and HsfA1 results in a marked synergistic effect suggesting that formation of homo- or heterocomplex of Hsfs might modulate the activity of heat stress genes.

These observations suggested the need to investigate if any significant difference exists in the protein-protein interactions between the tomato Hsfs. In this work tomato Hsfs were independently expressed in yeast (3.1.1) and the whole cell extract obtained from each strain was used to analyze their oligomerization behaviour (3.1.2) and to evaluate their protein interaction potential *in vitro* by means of pull down assays (3.1.3). In addition the successful use of the yeast two-hybrid system was extended to study details of the Hsf interactions *in vivo* (3.1.4). Finally their functional interactions were studied in tobacco protoplasts (3.1.5).

3.1.1 Expression of tomato Hsfs in yeast

Based on the functional replacement of yeast Hsf1 by an heterologous Hsf, it is possible to express and to study independently each tomato Hsf in yeast (Boscheinen et al., 1997). The *S. cerevisiae* strain (RSY4) with a chromosomal disruption of the *hsf1* gene (Wiederrecht et al., 1988) by site-specific insertion of the *his3* gene was supplemented with a plasmid encoding several tomato Hsfs (see Materials and methods). The Hsf types used throughout this work are shown in Table 3.1. Beside of wild type HsfA1 and HsfA2, mutated HsfA2 proteins with deletions at the C-terminus (HsfA2ΔC323 and HsfA2ΔC300) and in the oligomerization domain (HsfA2 Δ7/8,Δ323) and the M86 form of HsfA3 (aa 86-508), which is the dominant form found in tomato cell culture (Bharti et al., 2000), were used in this study.

Features of tomato Hsfs expressed in yeast have been already described by Boscheinen et al. (1997) and further characterized by Bharti et al. (2000). One of the major outcome was that all class A Hsfs can replace yeast Hsf1 in terms of its survival function whereas HsfB1 can not. Indeed differences in the activation domain of HsfB1 and not its DNA-binding ability or protein stability are responsible for this failure (Boscheinen et al., 1997).

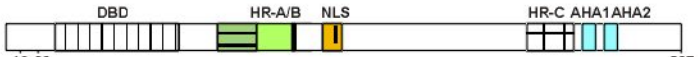
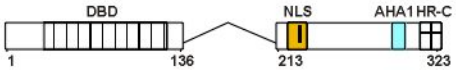
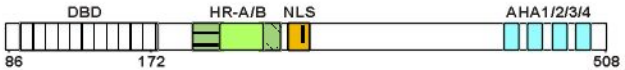
Hsf	Coding sequence	Structure	FOA survival	Code N.
HsfA1	aa 23-527		+	Ni 12
His_6-HsfA1	aa 23-527			Ni 85
HsfA2	aa 1-351		+	Ni 14
HsfA2ΔC323	aa 1-323		+	Ni 101
HsfA2ΔC300	aa 1-300		+	Ni 102
HsfA2Δ7/8,ΔC323	aa 1-136,VD, aa 213-323		+	Ni 136
HsfA3	aa M86-F508		+	SE 12
His_6-HsfA3	aa 172-508			SE 3
HsfB1	aa 1-301		-	Ni 13

Table 3.1 Overview of the different wild type and mutated Hsfs used in this study. Name, coding sequence, block diagram indicating the presence and position of the different functional modules of Hsfs are reported. Survival to FOA treatment of the respective yeast strains (FOA: survival (+) or nonsurvival (-) on 5-fluoroorotic acid plates at 28°C) and code number of the yeast and bacterial plasmids used (Code N.) are indicated. See Introduction and Figure 1.3 for further explanations about different functional modules of Hsf.

3.1.2 Oligomerization state of tomato Hsfs expressed in yeast

To investigate the oligomerization state of Hsfs in solution, whole cell extracts from yeast expressing HsfA1, HsfA2, HsfA3, or HsfB1 were cross-linked with increasing concentrations of ethylene glycol-bis (succinimidylsuccinate) (EGS). After reaction samples were separated by SDS-PAGE and analyzed by Western blotting. When HsfA1 extract reacts with increasing concentrations of EGS, the 67 kDa monomer is mainly converted into two sets of bands with apparent molecular sizes of 201 kDa and 402 kDa, which approximate to trimeric and hexameric forms of HsfA1 (Fig. 3.1 A). Similar results can be obtained with HsfA2. The 55 kDa monomer gives rise to cross-linked complexes of about 165 kDa and 330 kDa, sizes expected for a trimer and a hexamer (Fig. 3.1 B). Results with yeast extract expressing HsfA3 are less clear. The 52 kDa monomer gives rise to an approx. 156 kDa faint band corresponding to a trimer, and no higher protein complexes are visible (Fig. 3.1 C). Cross-linking of the HsfB1 extract (Fig. 3.1 D) causes the conversion of the 45 kDa monomer to a 90 kDa band corresponding to a putative dimer, as described by Boscheinen (1997). In general the loss of the monomer is associated with the concomitant appearance of cross-linked complexes, whose molecular weights are equivalent to multiples of the molecular weight of the monomer (samples 1 to 4). The less efficient transfer to nitrocellulose or reduced affinity to the antibodies due to EGS modifications might be responsible for the decreased intensity of the immune-detection (samples 5). These results demonstrate that all class A Hsfs can trimerize and eventually form higher oligomeric complexes as putative multiple of trimers whereas HsfB1 can dimerize.

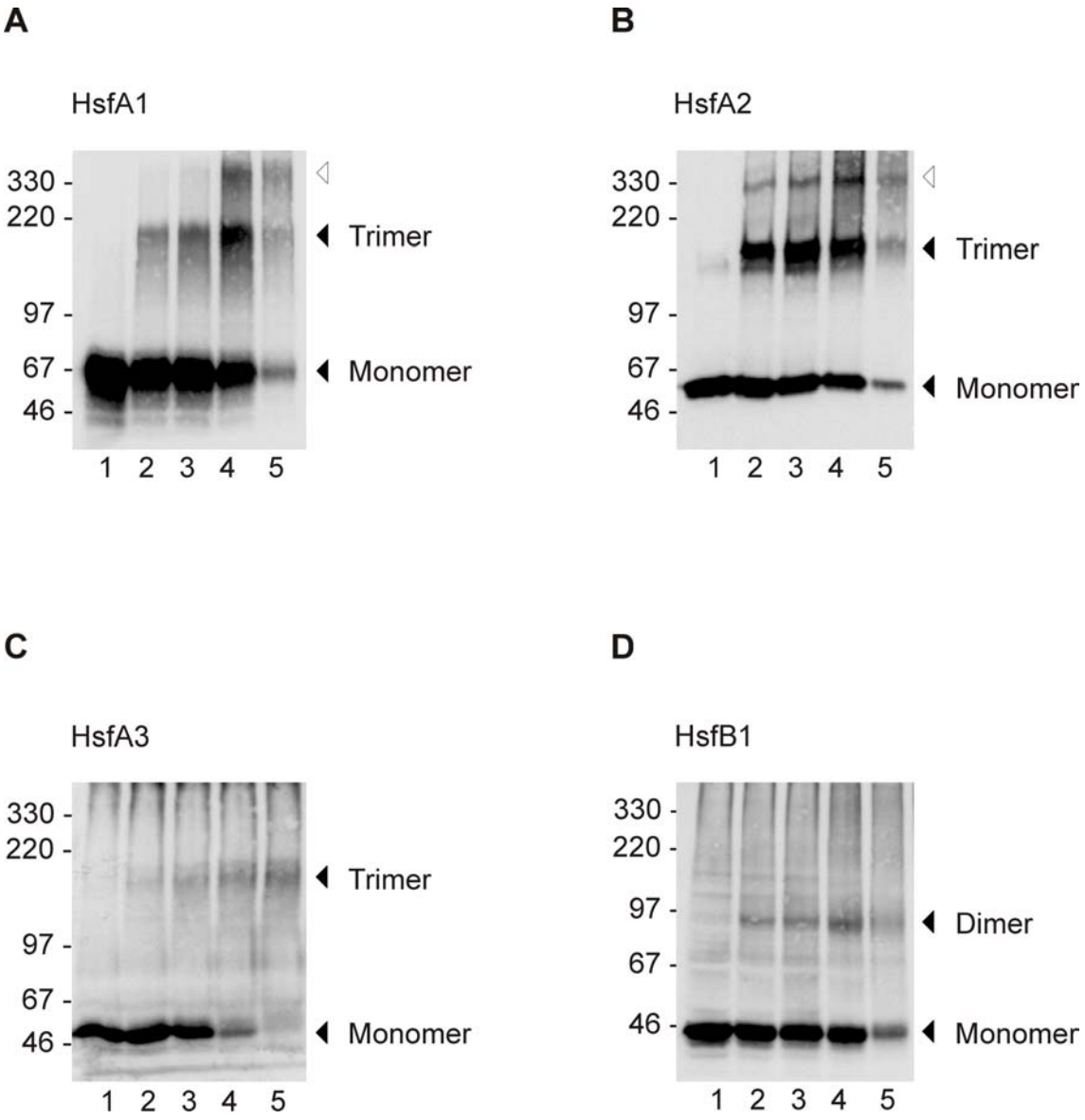


Figure 3.1 Oligomerization state of tomato Hsfs in yeast assessed by chemical cross-linking. HsfA1 (**A**), HsfA2 (**B**), HsfA3 (**C**) and HsfB1 (**D**) whole cell extract from yeast cells were chemically cross-linked with increasing concentrations of EGS and separated on 6 (or 8% in D) SDS-PAGE gels. Proteins were blotted to nitrocellulose and Hsfs were detected with anti-Hsf antibodies. Hsf polypeptides before (lane 1) and after reaction with EGS at concentration of 0.25 mM (lane 2), 0.5 mM (lane 3), 1 mM (lane 4) and 2 mM (lane 5). The solid arrowheads indicate the positions of the monomeric and trimeric or dimeric Hsf, whereas the open arrowheads point to other major cross-linked products. Molecular masses (in kDa) of markers are indicated on the left.

In vitro identification and characterization of interactions between members of the tomato Hsf family

To determine the existence and to further characterize the physical interaction between tomato Hsfs *in vitro* pull-down assays were carried out. Basically the pull-down assay was performed as follows: a purified recombinant His_6-tagged Hsf was incubated with a whole cell extract from a yeast strain expressing a different Hsf. After incubation, the proteins were rapidly purified on Ni-NTA agarose that strongly binds the His_6-tagged Hsf. Following extensive washes, bound proteins were eluted by boiling in SDS buffer, separated on 12% SDS-PAGE and identified by Western-blotting.

3.1.3.1 Class A Hsfs interact with each other but not with HsfB1

Two recombinant Hsfs with N-terminal His_6-tag were used: HsfA1 (aa 16-527) and HsfA3 (aa 172-508). Protein expression and purification procedures were carried out as described in Materials and methods. Yeast whole cell extracts were obtained from *S. cerevisiae* strains, which have been described in paragraph 3.1.1.

In order to verify that the pull-down assay was a suitable method to detect the interaction between tomato Hsfs, the first step was to reproduce the already known interaction between HsfA1 and HsfA2 and to test whether HsfB1 could also interact with HsfA1. The *in vitro* binding reactions of the pull-down assays were performed with three different amount of recombinant His_6-HsfA1 (0.1, 0.6 and 3.6 µg) and with increasing amounts of whole cell extract (50, 150 and 450 µg) containing HsfA2 or HsfB1. The result (Fig. 3.2 A) shows that the binding between HsfA2 and HsfA1 is specific and concentration dependent. Interestingly no binding of HsfB1 to HsfA1 is detectable at different concentrations tested (Fig. 3.2 B). For control, HsfA2 and HsfB1 extracts were also incubated with the Ni-NTA resin in absence of His-tagged HsfA1: the results excluded non-specific binding (data not shown).

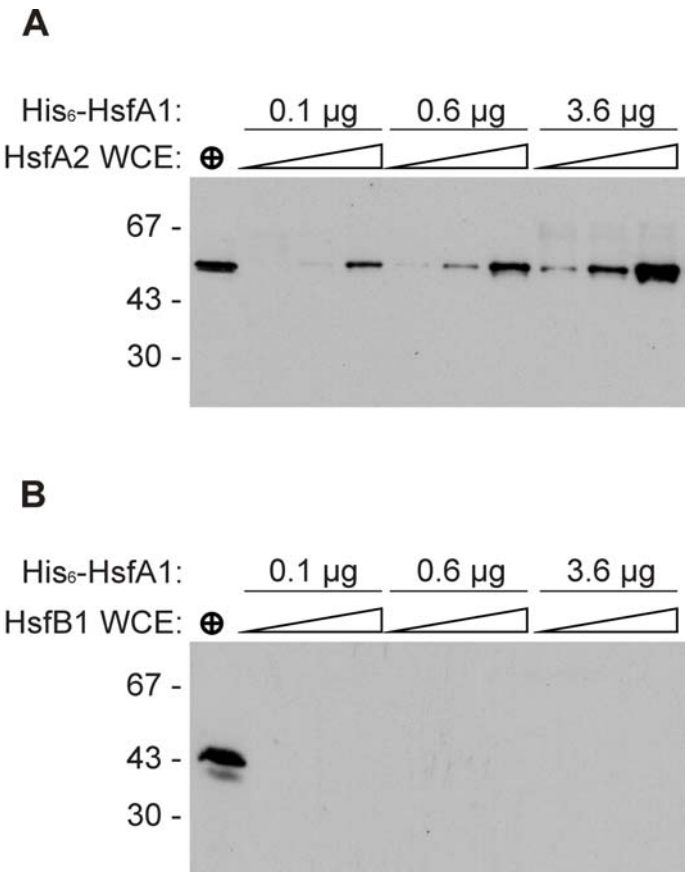


Figure 3.2 HsfA1 interacts with HsfA2 but not with HsfB1 (pull-down assay). Different amounts of recombinant His_6-HsfA1 were incubated with increasing amounts (50, 150 and 450 µg) of whole cell extract (WCE) from yeast strain expressing HsfA2 (**A**) or HsfB1(**B**). Bound proteins were eluted in sample buffer; half amount of the samples was analyzed by 12% SDS-PAGE and Western blotting with anti-HsfA2 (pep6) antibody (A) or anti-HsfB1 antibody (B). ⊕ indicates 10 µg of the corresponding yeast WCE which represents approx. 4.4% of the higher amount of WCE used in the binding reaction. Molecular masses (in kDa) of markers are indicated on the left.

Summarizing, these data indicate that the interaction between HsfA1 and HsfA2 can be documented: it is highly specific and stable as previously observed by Scharf et al. (1998b). Moreover it proves that HsfB1 can not interact with HsfA1. Therefore this approach can be extended to evaluate possible interactions between other members of the Hsf family.

The following set of *in vitro* pull-down assays was performed with HsfA3 as recombinant protein. In this case a constant amount of recombinant His_6-HsfA3 (3.6 µg) was incubated with increasing amounts of whole cell extract (50, 150 and 450 µg) containing HsfA1, or HsfA2, or HsfB1. Figure 3.3 shows that HsfA1 (A) and HsfA2 (B) are retained on the Ni-NTA resin by binding to the His_6-tagged HsfA3 and that this interaction is concentration dependent. Once more no HsfB1 complex can be retained on the Hsf-resin (C).

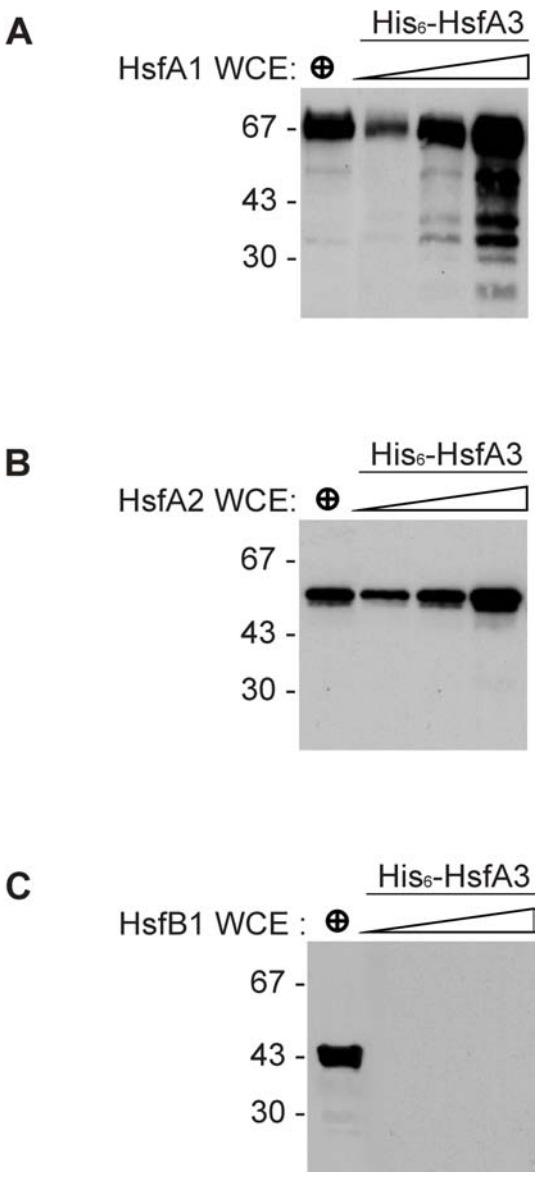


Figure 3.3 HsfA3 interacts with HsfA1 and HsfA2 but not with HsfB1 (pull-down assay). A constant amount of recombinant His_6-HsfA1 (3.6 µg) was incubated with increasing amounts (50, 150 and 450 µg) of WCE from yeast strain expressing HsfA1 (**A**), or HsfA2 (**B**), or HsfB1(**C**). Western analysis was performed with anti-HsfA1 antibody (A), or anti-HsfA2 (pep6) antibody (B), or anti-HsfB1 antibody (C). See legend to Figure 3.2 for further explanations.

3.1.3.2 The oligomerization domain (HR-A/B region) is required for interaction between class A Hsfs

To ascertain whether the C-terminal HR-C domain and/or the oligomerization domain (HR-A/B region) are essential for the interaction between class A Hsfs, His_6-tagged HsfA1 (3.6 µg) was incubated with yeast extract expressing HsfA2 proteins deleted at the C-terminus and/or in the oligomerization region. Three deletion forms of HsfA2 were used for the test: (B) HsfA2ΔC323 with a C-terminal deletion of 28 amino acids, (C) HsfA2ΔC300 with a C-terminal deletion of 50 amino acids and (D) HsfA2Δ7/8,ΔC323 with a C-terminal deletion of 28 amino acids and an internal deletion of 77 amino acids in the oligomerization domain. The results summarized in Figure 3.4 demonstrate that the interaction between HsfA1 and HsfA2 is lost as soon as the oligomerization domain of HsfA2 is deleted, while the C-terminal deletions of HsfA2 do not affect the stability and strength of the *in vitro* protein interactions.

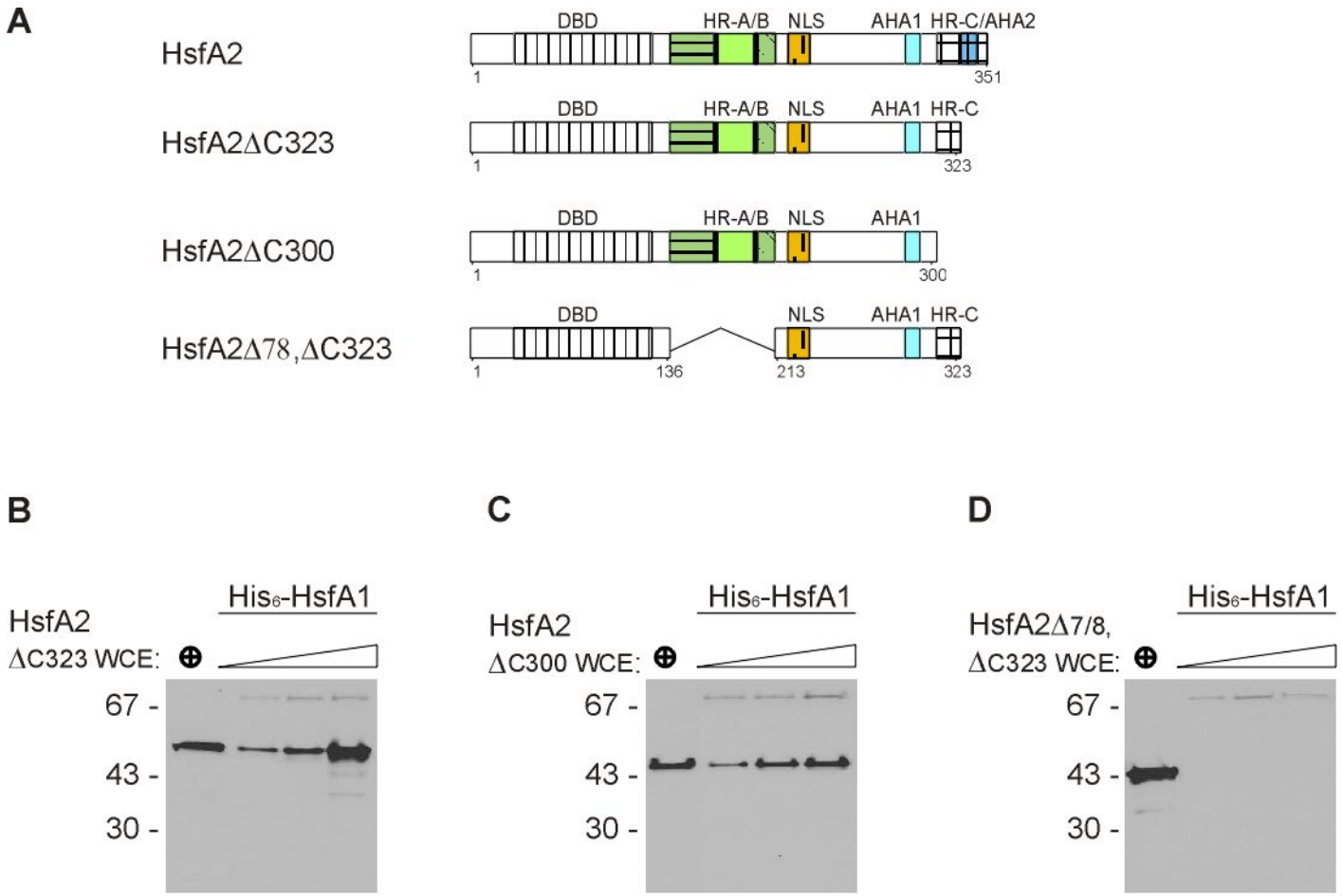


Figure 3.4 HsfA1 and HsfA2 interact via the oligomerization domain (pull-down assay). A schematic representation of wild type and mutated HsfA2 proteins is shown in (**A**). A constant amount of recombinant His_6-tagged HsfA1 (3.6 µg) was incubated with increasing amounts (50, 150 and 450 µg) of WCE from yeast strain expressing HsfA2ΔC323 (**B**), or HsfA2ΔC300 (**C**), or HsfA2Δ7/8,ΔC323 (**D**). Western analysis was performed with anti-HsfA2 antibody; the upper faint band is an antibody cross-reaction to recombinant His_6-tagged HsfA1. See legend to Figure 3.2 for further explanations.

3.1.4 *In vivo* identification and characterization of interactions between members of the tomato Hsf family

In order to further characterize the interactions between members of the tomato Hsf family the yeast two-hybrid system was chosen because it can identify pairs of proteins that physically interact with each other *in vivo*. This system also permits detection of weak and transient interactions. The following prerequisites are indispensable for a successful application of the yeast two-hybrid system:

- The relevant portion of the Hsfs must be fused in-frame with those encoding the Gal4 DNA-binding domain (aa 1-147, Gal4 BD) or the Gal4 transcription activation domain (aa 768-881, Gal4 AD) in the yeast two-hybrid vectors (Stratagene); proteins fused to the Gal4 BD are referred to as baits, whereas those fused to the Gal4 AD are referred to as preys.
- The interaction of the two hybrid proteins in yeast reconstitutes a transcription factor able to activate transcription of the *his3* gene under the control of the *gal4* promoter. In case of positive interaction, yeast survival under conditions of histidine starvation can be directly correlated to the extend to which the *his3* promoter is activated, providing a direct assessment of the efficiency of interaction between the bait and the prey.
- Essential for the function as bait is the inability to activate *his3* reporter gene expression without protein interaction. Therefore use of a transcriptional activator as bait requires to delete or mutate part of the protein responsible for the function as transcriptional activator. Hence HsfA1 (aa 23-447) and HsfA3 (aa 177-421) baits have a C-terminal deletion in the activation domain, whereas HsfA2 (aa 98-351; W297>A, W337>E, and L341>A) carries point mutations in the AHA motifs of the activation domain. However, HsfA2 and HsfA3 baits have still weak transactivating potential. To circumvent this situation the 3-amino-1,2,4-triazole (3-AT), an inhibitor of the dehydratase encoded by the *his3* gene, was added at the concentration of 5 mM to the selective growth media wherever these two baits were used. On the contrary, HsfB1 bait is given by the full cDNA coding sequence (aa 1-301) since HsB1 has no detectable activator potential in yeast.

Figure 3.5 shows a schematic representation of protein fragments of HsfA1, HsfA2, HsfA3 and HsfB1 used in bait and prey position: presence and position of relevant functional domains and amino acid residues are indicated. Plasmid numbers are given in brackets.

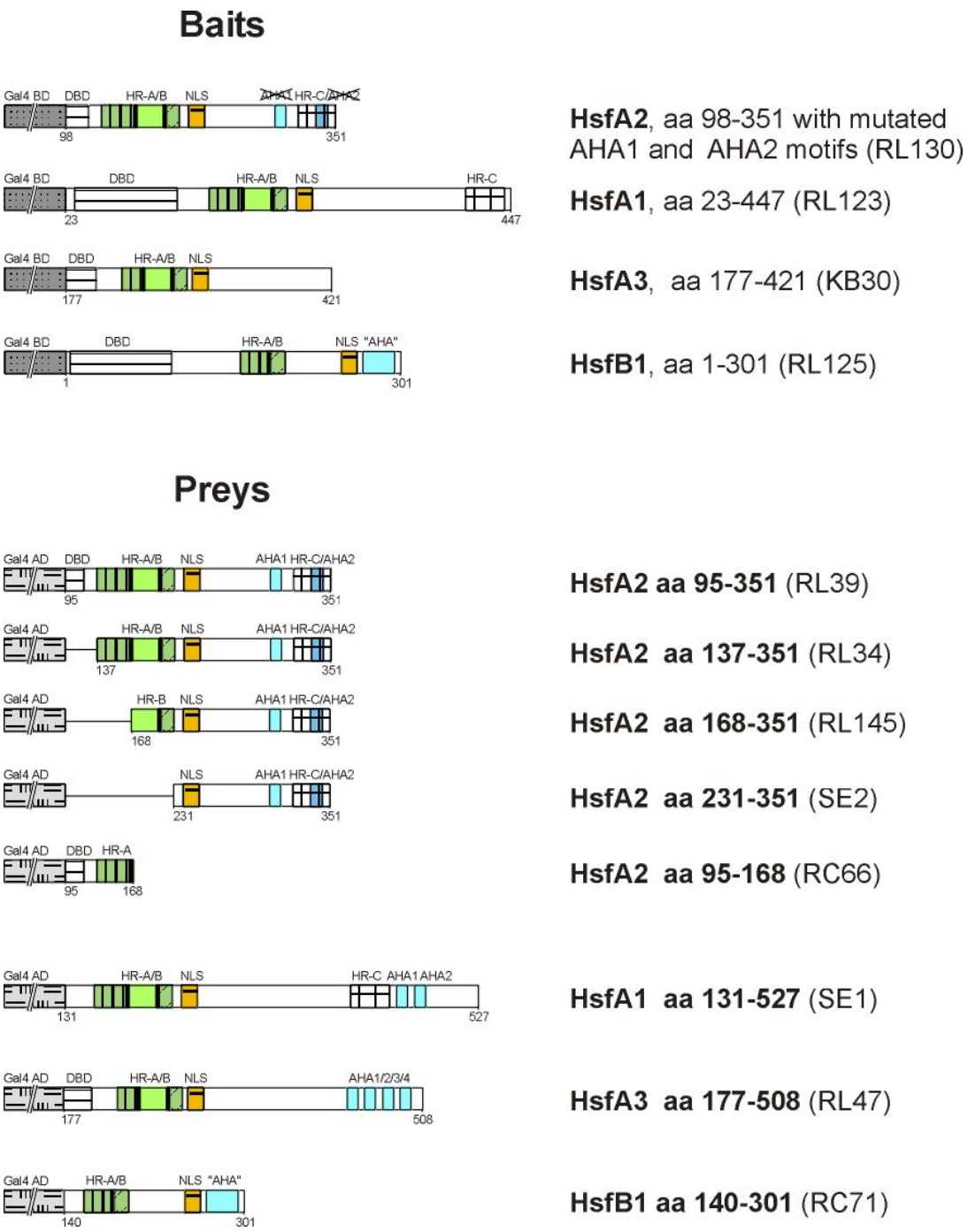
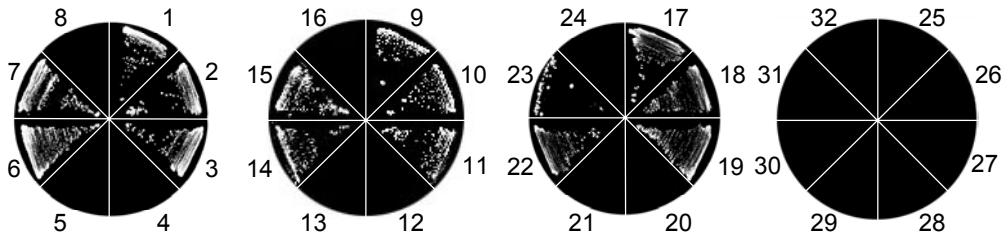


Figure 3.5 Hsf baits and preys used in the two-hybrid assay. The indicated Hsf proteins are fused either to the yeast Gal4 DNA-binding domain (aa 1-147, Gal4 BD) and correspond to baits, or to the yeast Gal4 activation domain (aa 768-881, Gal4 AD) and correspond to preys. Schematic representation of the proteins, relevant amino acid residues, nomenclature of baits and preys (bold typed) and plasmids code number (in bracket) are given. See Introduction and Figure 1.3 for further explanations about different functional modules of Hsf.

3.1.4.1 Interactions of class A Hsfs in the yeast two-hybrid system

The three bait constructs encoding fragments of HsfA1, HsfA2 and HsfA3 were combined with the prey constructs encoding different parts of the three Hsfs (see table in Fig. 3.6 for details). Interactions between baits and preys were assessed qualitatively, determining the *his3* reporter gene activity in YRG-2 cells by observing growth of cotransformants on His-free media.

In Figure 3.6 the performed cotransformations are reported in a table and the growth tests (streaks) on solid selective media show that all Hsfs can interact with each other. These interactions can occur as long as part of the oligomerization domain (linker and HR-B region) is present. These results also show that, whereas HsfA1 and HsfA2 present strong interactions in both homo- and heterodimeric combinations, only the heterodimeric interactions of HsfA3 is evident. Indeed, HsfA3 homodimeric interaction results in a reduced yeast growth (sample 23): its low ability to homooligomerize was also previously observed *in vitro* (Fig. 3.1 C)



Bait \ Prey	HsfA2 aa 95-351	HsfA2 aa 137-351	HsfA2 aa 168-351	HsfA2 aa 231-351	HsfA2 aa 95-168	HsfA1 aa 131-527	HsfA3 aa 177-508	Gal4 AD
HsfA2	1	2	3	4	5	6	7	8
HsfA1	9	10	11	12	13	14	15	16
HsfA3	17	18	19	20	21	22	23	24
Gal4 BD	25	26	27	28	29	30	31	32

Figure 3.6 *In vivo* interactions between class A Hsfs (two-hybrid assay). The indicated Hsf proteins, fused to the yeast Gal4 DNA-binding domain and to the yeast Gal4 activation domain (see Fig. 3.5 for further explanations) were coexpressed into the YRG-2 yeast strain according to the table. As negative control, each bait or prey was also coexpressed with Gal4 AD (samples 8, 16 and 24) and Gal4 BD (samples 25-32). Protein interaction is indicated by growth on minimal media lacking histidine, leucine and tryptophan (and eventually supplemented with 3-AT). The growth behaviour shown by the pictures of the agar plates is summarized in the table; green shaded areas highlight the positive interactions.

3.1.4.2 Functional analysis of the oligomerization domain by mutants

The above reported results with different deleted forms of HsfA2 (Fig. 3.6) indicate that the linker and the HR-B region of the oligomerization domain are sufficient for interaction. This minimal interaction domain encompasses amino acid residues 168-201. Sequence alignment of the oligomerization domain (HR-A/B region) of class A Hsfs shows that this region is characterized by two overlapping hydrophobic heptad repeats (Fig. 3.7, open circles: **o**, and stars: *****) which suggest a coiled-coil structure. However, it is not immediately clear from the sequence whether the HR-B forms an independent coiled-coil structure and the linker is a connecting region with the HR-A region, or the linker and the HR-B region form a continuous coiled-coil structure.

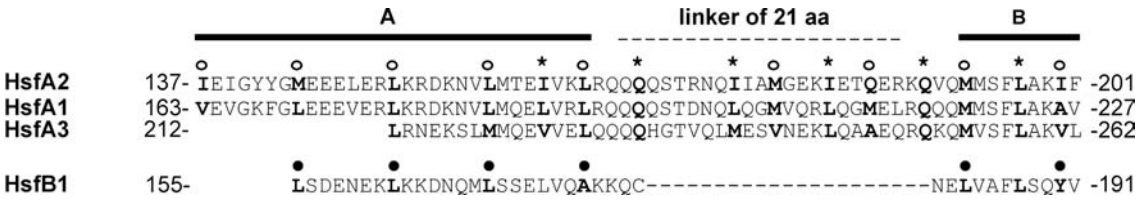


Figure 3.7 Sequence alignment of the HR-A/B region from tomato Hsfs. Protein sequences are given in one-letter code and labeled with the protein name to the left; numbers denote the first and the last amino acid residues in each region. The heptad repeat positions are marked; two overlapping repeat patterns are found in class A Hsfs (open circles and stars respectively), whereas HsfB1 has a single continuous pattern (closed circles). Amino acid residues that define the beginning of each heptad are bold typed. "**-**" indicates a gap introduced to optimize the alignment.

Two approaches were followed to investigate the conserved amino acid residues in the linker and HR-B region with respect to their role for protein interaction:

- Change of the highly conserved hydrophobic residues of the HR-B region in order to disrupt the surface exposed residues involved in putative hydrophobic interactions. Specific double alanine amino acid substitutions were introduced at positions 193/194 (MM>AA), or 196/197 (FL>AA) or 200/201 (IF>AA). Alanine was chosen for substitution because it is a hydrophobic amino acid unable to provide side-chain interactions involving atoms beyond the β-carbon.
- Change of the length of the linker region between the HR-A and HR-B region in order to disrupt the continuity of the heptad pattern of hydrophobic residues as a basis for a coiled-coil structure. One approach was to insert 4 amino acids (ARGA, mutation HR+4) or 6 amino acids (ARGVGA, mutation HR+6) between residues 168 and 169. Hypothetically these mutations would shift the position of the highly conserved hydrophobic residues in a helix (Fig. 3.8 B). Another approach was to delete residues from amino acid 169 to amino acid 190 (mutation HR-21) to create a hydrophobic repeat pattern similar to that of the oligomerization domain of class B Hsfs, e.g. HsfB1 (Fig. 3.8 C).

Nomenclature of the mutations made for this study and sequence details are presented in Figure 3.8 A .

Figure 3.8 Details of mutations in the HR-B and linker region of HsfA2. See Figure on the next page. **(A)** In the HR-B region of HsfA2 the amino acid residues considered for mutations are underlined in red, whereas in the linker region red arrows indicate the positions after which amino acid residues were introduced (after the aa 168) or deleted (from aa 169 to 191). Nomenclature of mutations made for this study and code number of plasmids used (in brackets) are reported. (**B** and **C**) Helical wheel representation of wild type and mutated HsfA2 proteins; HsfB1 is also shown for comparison. The seven amino acid residues of the heptad repeat are referred to by letters “a” to “g” according standard nomenclature. Mutations (substitution, extension and deletion) are underlined or colored in red. See Figure 3.7 for further explanations.

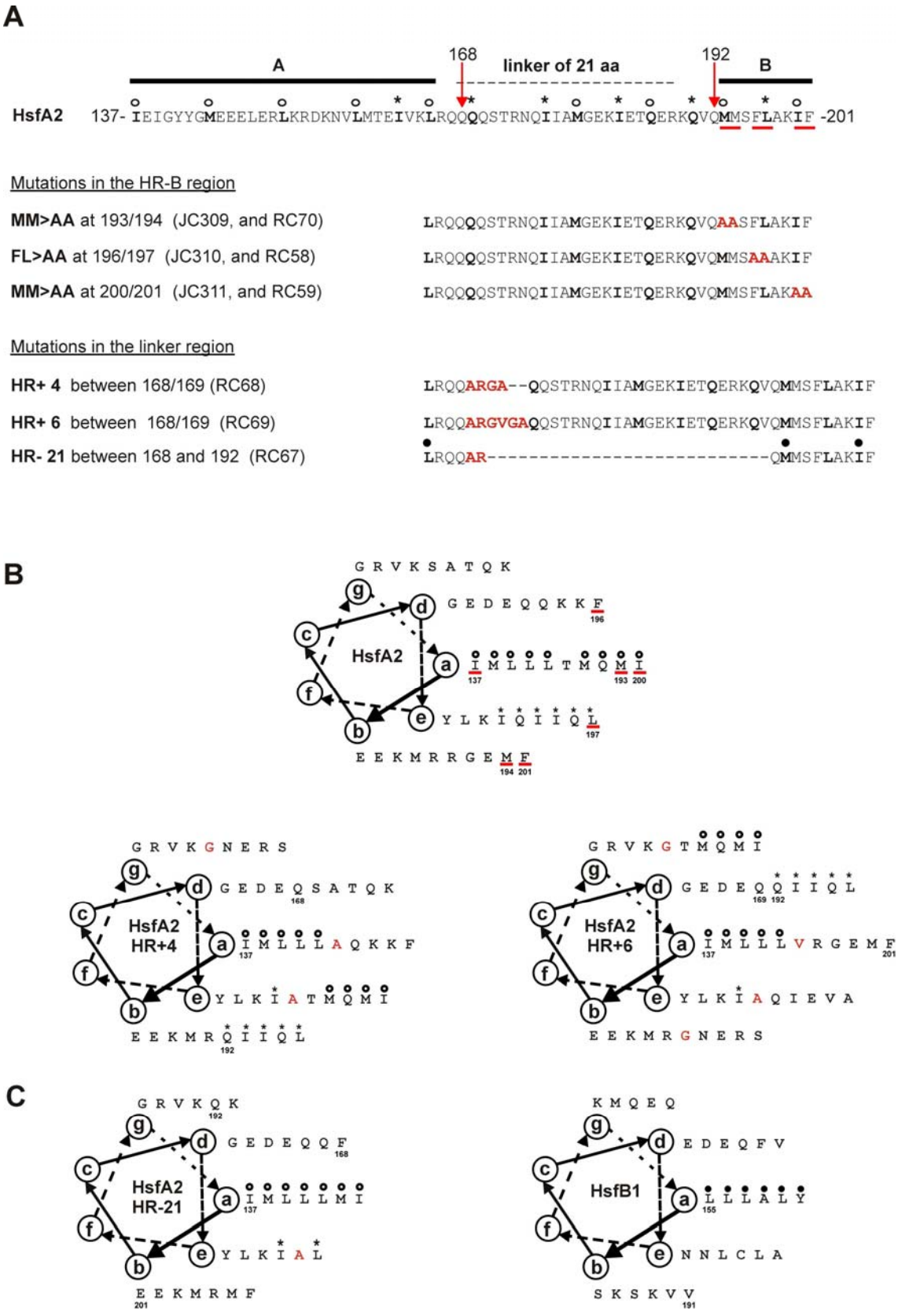
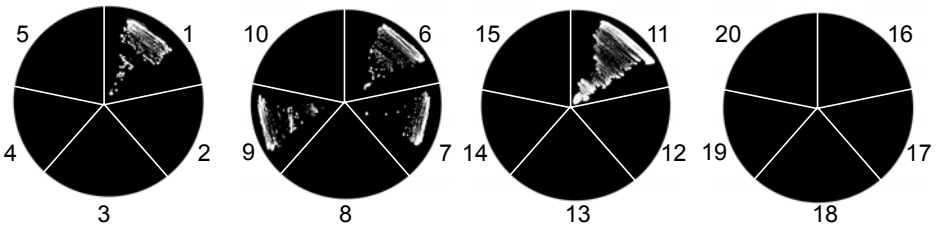


Figure 3.8 Details of mutations in the HR-B and linker region of HsfA2. See legend on the previous page.

First, each of the double alanine mutation was introduced in the background of HsfA2ΔN168-351 prey (Janista C., diploma work, 2000) and these preys were tested for interaction with HsfA2, HsfA1 and HsfA3 baits. The results in Figure 3.9 A show that HsfA2 (samples 2, 3 and 4) and HsfA3 (samples 12, 13 and 14) can not interact with these mutated preys. HsfA1 interaction was impaired only by the mutation FL>AA (sample 8) but not by the other two (samples 7 and 9). To confirm these results all these mutations were also introduced in the background of HsfA2 (aa 98-351) in bait position. As described before these baits were tested for interaction with C-terminal fragments of HsfA2, HsfA1 and HsfA3 in prey position (Fig. 3.9 B). The results for HsfA1 and HsfA2 preys are similar to those in Figure 3.9 A. It is therefore surprising that the interactions between mutated HsfA2 (bait positions) and HsfA3 (prey position) are not affected (samples 7, 11 and 15). In a second step the prey constructs encoding HsfA2 with a deletion of 21 amino acid residues or an insertion of 4 or 6 amino acid residues between the HR-A and HR-B regions were cotransformed with the Hsf baits as indicated in Figure 3.10. One of the main outcomes of this approach is that the mutated protein with an oligomerization domain resembling that of HsfB1 does not interact with any of the four baits, including HsfB1. In contrast, the two insertions altering the heptad pattern between HR-A and HR-B have no effects.

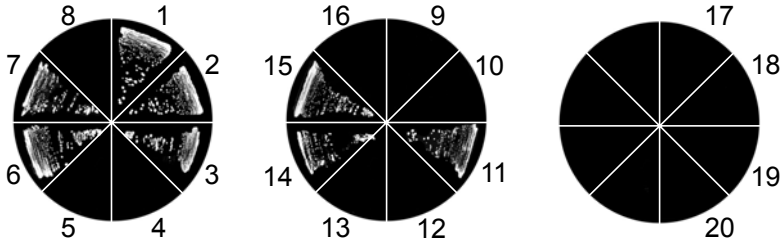
All these findings are compatible with the hypothesis that only HR-B is the essential part for protein interaction and its highly conserved amino acid residues contribute differently to the specificity of homo- or heterodimeric protein interactions.

A



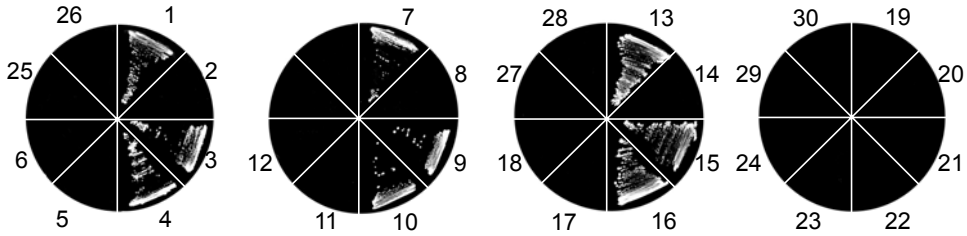
Bait \ Prey	HsfA2 aa 168-351	HsfA2 aa 168-351, MM>AA	HsfA2 aa 168-351, FL>AA	HsfA2 aa 168-351, IF>AA	Gal4 AD
HsfA2	1	2	3	4	5
HsfA1	6	7	8	9	10
HsfA3	11	12	13	14	15
Gal4 BD	16	17	18	19	20

B



Bait \ Prey	HsfA2 aa 168-351	HsfA1 aa 131-527	HsfA3 aa 177-508	Gal4 AD
HsfA2	1	2	3	4
HsfA2, MM>AA	5	6	7	8
HsfA2, FL>AA	9	10	11	12
HsfA2, IF>AA	13	14	15	16
Gal4 BD	17	18	19	20

Figure 3.9 ***In vivo*** **interactions between class A Hsfs and HR-B mutants of HsfA2 (two-hybrid assay).** The HR-B mutations (MM>AA, FL>AA and IF>AA; see Fig. 3.8) were tested both in prey position in the background of the HsfA2dN168-351 protein (**A**) and in bait position in the background of the HsfA2dN98-351 protein (**B**) for protein interactions. See legend to Figure 3.6 for further explanations.



Bait \ Prey	HsfA2 aa 95-351	HsfA2 aa 95-351, HR- 21	HsfA2 aa 95-351, HR+ 4	HsfA2 aa 95-351, HR+ 6	HsfB1 aa 140-301	Gal4 AD
HsfA2	1	2	3	4	5	6
HsfA1	7	8	9	10	11	12
HsfA3	13	14	15	16	17	18
HsfB1	19	20	21	22	23	24
Gal4 BD	25	26	27	28	29	30

Figure 3.10 ***In vivo*** **interactions between Hsfs and linker mutants of HsfA2 (two-hybrid assay).** The linker mutations (HR+4, HR+6 and HR-21; see Fig. 3.8) were tested in prey position in the background of the HsfA2dN95-351 protein. See legend to Figure 3.6 for further explanations.

3.1.5 Functional analysis of tomato Hsfs in tobacco protoplasts

We investigated further if there is any correlation between the formation and the transcriptional activity of Hsf homo- and heterocomplexes since previous results suggested that the heterocomplexes might represent the most stable and transcriptionally active forms (Scharf et al., 1998b).

A suitable system for the functional analysis of tomato Hsfs is the transient expression assay in tobacco mesophyll protoplasts by cotransformation of Hsf expression plasmids together with an appropriate Hsf-dependent *gus* reporter (Treuter et al., 1993; Döring et al., 2000). As shown in Figure 3.11 A, the overall transactivation potential of the wild-type HsfA2 (A2) is higher under heat stress than control conditions. Indeed HsfA2 mainly localizes in the cytoplasm and thereby it functions poorly as transcription activator (Lyck et al., 1997). The mutated HsfA2 proteins (see Fig. 3.8 for HR-B and linker mutations, and

Appendix C) do not display real different abilities in the activation of the Hsf-dependent reporter gene although weak changes are observed. HsfA2 proteins with mutations in the HR-B region (A2MM>AA, A2FL>AA, A2IF>AA) activate more than the wild type HsfA2 even under control conditions, whereas HsfA2 proteins with deletion of the linker region (A2HR-21) or the oligomerization domain (A2Δ7/8) seem to be less active. The GUS activity measured in sample transformed with the *gus* reporter plasmid alone (endog.) reflects the activity of endogenous Hsfs. Therefore the low GUS activity measured in samples transformed with A2HR+4 or A2Δ7/8 under control conditions probably indicates a competition between the HsfA2 proteins and the endogenous Hsfs for binding to HSE sites on the Hsf-dependent reporter gene since Western analysis assessed that the wild type and the mutated forms of HsfA2 were expressed at comparable level (data not shown).

Figure 3.11 B shows GUS activity levels in protoplasts coexpressing several Hsfs. Previous results have shown that the coexpression of HsfA2 and HsfA1 leads to an enhanced transcriptional activity due to nuclear localization of HsfA2 via protein interaction with HsfA1 (Scharf et al., 1998b). Indeed GUS activity in protoplasts coexpressing HsfA2 and HsfA1 (A1/A2) is higher than the one observed with HsfA1 or HsfA2 alone. This effect is more than additive. Once more the mutated forms of HsfA2 modulate the transcriptional activity weakly and differently. Surprisingly these activities seem not that much dependent on the interaction with HsfA1: enhanced activity is also observed between proteins that have been shown not to interact with each other (see Fig 3.9 for A1/A2FL>AA, Fig. 3.10 for A1/A2HR-21 and Fig 3.4 for A1/A2Δ7/8,ΔC323). However, the mutations in the linker region and the deletion of the oligomerization domain of HsfA2 have a general negative effect on the transcriptional activity. These results also show that the coexpression of HsfA2 and HsfA3 (A2/A3) can determine a GUS activity lower than the one measured in sample coexpressing HsfA2 and HsfA1. Moreover the coexpression of HsfA3 with each mutated form of HsfA2 does not lead to any relevant variations (data not shown). Finally each class A Hsf was also coexpressed with HsfB1: interestingly HsfA1 together with HsfB1 (A1/B1) shows a high synergistic activation of the Hsf-dependent reporter gene, despite the lack of a direct interaction between these two proteins (Fig. 3.2).

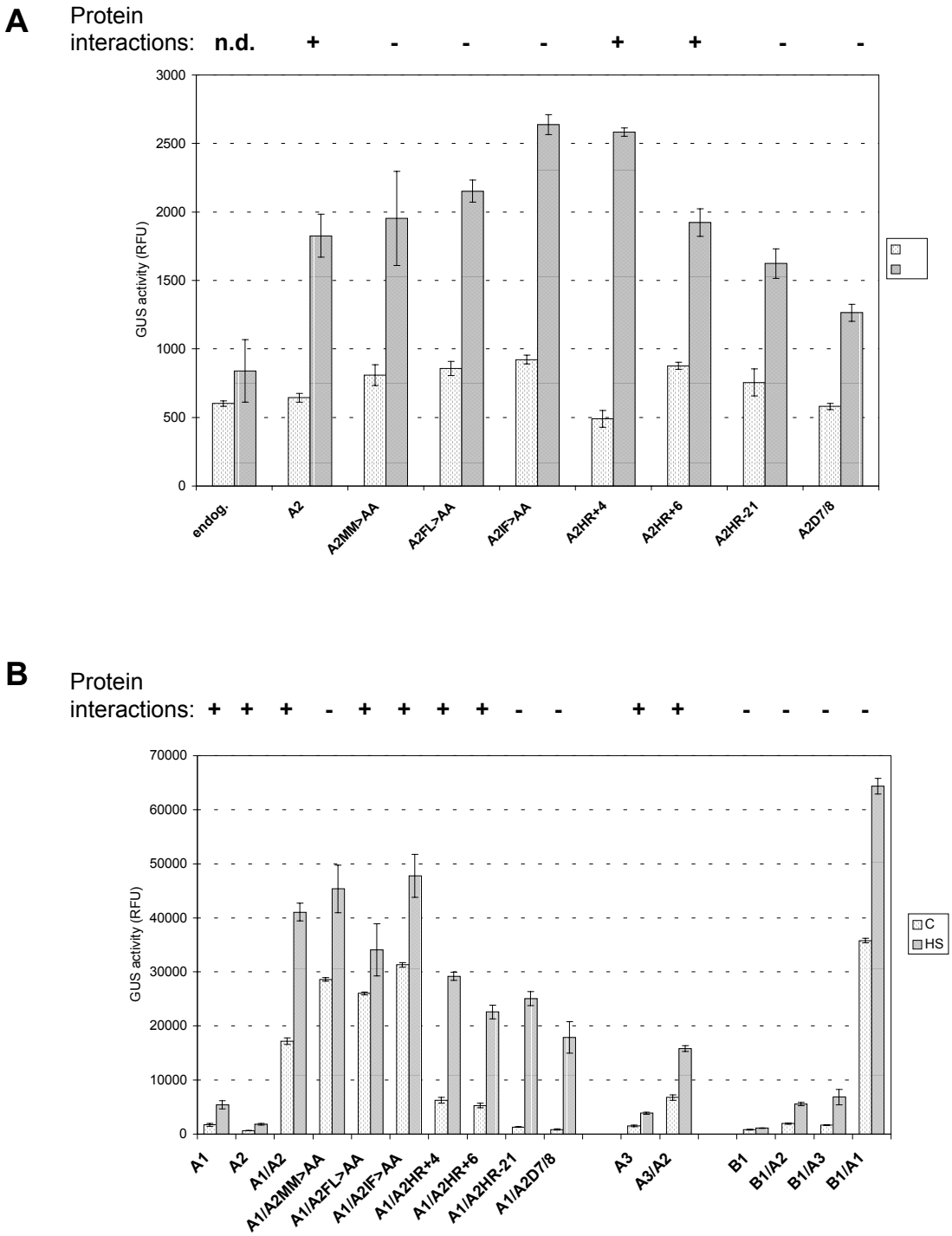


Figure 3.11 Functional analysis of wild-type and mutated HsfA2 proteins (A) and their coexpression with different tomato Hsfs (B) (GUS activity assay). Tobacco protoplasts were transformed with the *phsp17*gus* reporter and with the indicated Hsf expression plasmids (4 µg was the total amount of DNA; see Appendix C for plasmid maps). After incubation for 6 h at 25°C in dim light; half of the samples were heated to 35°C for 2 h and then left at 25°C for 2 more hours (HS: heat stress), whereas the other half was maintained at 25°C (C: control). Results are the mean of three independent transformations. endog.: activity of endogenous Hsfs. Protein interactions: protein interactions between tomato Hsfs observed during this work; n.d.: not determined. Histograms are reported with different scales.

These results demonstrate that the overall transactivation potentials of Hsfs are not dependent only on their oligomerization state (as monomers or homo- and heterocomplexes) in these experimental conditions. Indeed mutations that abolish protein interactions between subunits of HsfA2 (e.g. A2IF>AA and A2Δ7/8) as well as proteins that do not interact with each other (e.g. class A Hsfs and HsfB1) can still differently activate a Hsf-dependent *gus* reporter. Therefore, although oligomerization state of mutated HsfA2 proteins (see 4.1.2) nuclear localization and intrinsic activator potential of each Hsf (Scharf et al., 1998b; Döring et al., 2000; Bharti et al., 2000) should be taken to account for a correct evaluation of these data, so far the results suggest that protein-protein interactions at DNA level between Hsfs, binding at adjacent HSE sites, play a determinant role in the activation of a Hsf-dependent gene.

3.1.6 The oligomerization states of Hsfs: monomer-to-trimer transitions, dimers and constitutive trimers

The oligomerization state of Hsfs have been analyzed in several studies. In general studies which have examined metazoan Hsfs have provided information on the monomer-to-trimer transition (Rabindran et al., 1993; Westwood and Wu, 1993; Zuo et al., 1994): under non stress conditions, Hsf is monomeric and is located in the cytoplasm, while upon stress, the protein trimerizes and translocates to the nucleus (Baler et al., 1993; Sarge et al., 1993). Hence the heat stress induced transition between monomeric, non DNA-binding and inactive Hsf, to the trimeric, DNA-binding and active form (Fig. 1.5.) is an intrinsic part of the activation-deactivation cycle of Hsfs. Conversely, yeast Hsf is thought to be a constitutive trimer with intrinsic DNA-binding ability and located in the nucleus under all conditions (Jakobsen and Pelham, 1991; Liu et al., 1997). The oligomerization state and its dynamic changes are maintained when Hsfs are studied in an heterologous model system, e.g. human Hsfs present a transition from monomers to trimers when expressed in yeast or in *Xenopus* oocytes (Liu et al., 1997 and 1999; Baler et al., 1993). Moreover, yeast has already been shown to be a successful system to analyze tomato Hsfs (Boscheinen et al., 1997).

Interestingly, as reported in paragraph 3.1.2, all tomato class A Hsfs seem to be constitutive trimers that might have different stability and ability to form higher homooligomeric complexes (compare HsfA3 to HsfA1 and HsfA2 states in Fig. 3.1). Although differences in the yield of cross-linked products might also be related to the reactivity of the cross-linking reagent to the Hsf in analysis as well as to its relative concentration in solution, these results indicate the absence of monomeric forms of tomato class A Hsfs. Indeed, it was never possible to demonstrate the existence of monomeric Hsfs forms in tomato cells or in tobacco protoplasts during Hsfs transient expression (unpublished results from our group). Moreover no real evidence for heat stress dependent changes of the oligomerization state of tomato Hsfs has ever been observed, as instead reported for *Drosophila* and human Hsfs (unpublished results from our group; Westwood et al., 1991; Zuo et al., 1994).

The dimeric state of tomato HsfB1 was also shown by other assays (EMSA, gelfiltration and isotherm titration calorimetric analyses - Boscheinen O., PhD thesis, 1997; Kunert O., PhD thesis, 1997). However, its oligomerization state was analyzed only in presence of Hsf-sequence specific DNA oligonucleotides and the influence of DNA on the homodimer formation could not be excluded. Furthermore, Hsf dimeric states have also been observed for human Hsf2 (Sistonen et al., 1994) and for chicken HsfA3 (Nakai et al., 1995). The induction of their DNA-binding properties is accompanied by a transition from an inert dimeric state to an activated trimer.

3.1.7 Class A Hsfs interact with each other via the oligomerization domain: linker and HR-B regions are the minimal domain required

The oligomerization domain has been already shown to be responsible for homotrimerization of Hsfs (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992, Zuo et al., 1994; Boscheinen et al., 1997).

Interestingly this study shows that the oligomerization domain is involved not only in the homologous but also in the heterologous protein interactions between tomato class A Hsfs (Fig. 3.4 and 3.6). In particular, by testing deleted forms of tomato HsfA2, the protein interactions between class A Hsfs can occur

as long as the linker and the HR-B regions of the oligomerization domain are present (Fig. 3.6). The results also indicate that the HR-A region of the oligomerization domain and the HR-C region in the C-terminal part of the protein do not play a relevant role in the protein interactions since their presence is not sufficient to give a positive interaction and their deletions do not affect the stability and strength of the protein interactions (Fig. 3.4 and 3.6).

These data are evidently observed *in vitro* as well as *in vivo* (3.1.3 and 3.1.4) and are mainly in agreement with previous studies about the homotrimerization of Hsf (Sorger and Nelson, 1989; Clos et al., 1990; Rabindran et al., 1991; Peteranderl and Nelson, 1992; Zuo et al., 1994; Boscheinen et al., 1997). Moreover, they provide full evidence for physical interaction between different Hsfs, although limited to tomato class A Hsfs, in agreement with observations by Scharf et al. (1998b).

Up to date there is no other evidence for a physical interaction between Hsfs. It is reported that differences in the length and amino acid sequence of the linker region, as well as variations in other positions of the heptad repeat that contribute to pattern specificity (Cohen and Parry, 1994), probably account for the absence of the mixed trimers and of the trimer–trimer interaction when different Hsfs are coexpressed in the same cells (Clos et al., 1993; Rabindran et al., 1993; Sistonen et al., 1994; Nakai at al., 1995). These observations might also be valid for the lack of interactions between class A Hsfs and HsfB1 (Fig. 3.2 and 3.3).

3.1.8 The linker and HR-B regions have a diverse role in class A Hsfs interactions

Several suggestions to understand the principles that determine the ability of class A Hsfs to interact with each other can be obtained by structural studies on the oligomerization domain of Hsf, by principles that determine the specificity of pairing of coiled coils, and by the functional analysis of the oligomerization domain of HsfA2 by mutants.

Recent biochemical and biophysical characterization of the trimerization domain from *K. lactis* Hsf has shown that it is a highly elongated coiled-coil

structure: three parallel strands are in register along the long axis of the coiled-coil and the C-termini of the subunits are in close proximity (Peteranderl et al., 1999). These studies also report that a potential break in the coiled-coil region is located between the HR-A and HR-B region. Moreover, by analyzing the two regions as isolated polypeptides, Peteranderl and coworkers (1999) have postulated that mainly the HR-B region contributes to trimer formation whereas the HR-A region provides major stability to the complete domain.

The coiled-coil domain is a highly versatile protein folding motifs. Principles that determine its specificity and oligomerization states are described in several recent reviews (Lupas, 1996; Burkhard et al., 2001). The fold of a coiled-coil motif is determined by a simple pattern of amino acid residues, in which there is a characteristic heptad repeat, designated by the letters **a** to **g**. The first and fourth positions of the repeat, the **a** and **d,** form the interior of the interacting strands of the coiled-coil (helix interface) whereas the residues at the other positions form the solvent-exposed part. Moreover interhelical electrostatic interactions between residues at the **g** position and the **e** position of the succeeding heptad on the adjacent helix have been observed to promote specificity (Baxevanis and Vinson, 1993). The interface of the helices generally consists of hydrophobic residues, nevertheless many naturally occurring coiled-coil proteins also contain buried polar residues (Harbury et al., 1993; Lumb and Kim, 1995; Hendsch et al., 1996). Residues at the **a** and **d** positions have profound effects on the oligomerization states of coiled-coils (Harbury et al., 1993 and 1994; Zhu et al., 1993). In this regard remarkable progress has been made by the analyses of the GCN4 leucine zipper mutants that have shown concerted switches between two-, three-, and four-stranded coiled-coil structures. The switch is governed by packing constraints imposed by distinct local geometry at the **a** and **d** positions.

3.1.8.1 The linker ensures flexibility to the HR-B region for productive protein interactions

Interestingly the mutations in the linker region of HsfA2 show that the protein interactions are not impaired by the insertion of 4- or 6- residues (mutations HR+4 and HR+6) (Fig. 3.10). Therefore it seems likely that the extra-amino acids introduced do not shift the positions of any relevant residues (e.g. at **a** and **d** positions of the helix), which determine the helix interacting surface, but rather they localize in a region that does not have any impact on the structure, suggesting that the HR-B region keeps an unperturbed coiled-coil structure.

Coiled-coils have often discontinuities in the structures, which are determined by skip residues (extra residues in the heptad pattern, e.g. in the DNA polymerase I), stutters (omission of three or four residues, e.g. in the influenza hemagglutinin), and even short loops (e.g. in the influenza hemagglutinin or fibritin, a homotrimeric structural protein of bacteriophage T4). The effect of these discontinuities do not perturb the overall coiled-coil structure significantly (Lupas, 1996; Tao et al., 1997; Burkhard et al., 2001).

The presence of the linker is necessary: protein interactions are lost when the linker is deleted (mutation HR-21), although the HR-A and HR-B region would theoretically keep a coiled-coil motif as in the wild type protein (Fig 3.10 and 3.8). It follows that the highly conserved amino acids (all of them or only those at the N-terminal part) of the linker region might not contribute to form the coiled-coil structure of the oligomerization domain and might not provide an interacting surface for the protein interaction between class A Hsfs. Instead they have an essential role in forming an unstructured spacing region which provides flexibility to the oligomerization domain. This suggestion is supported by the fact that secondary structure analyses do not predict the linker region between the HR-A and HR-B regions of the *K. lactis* trimerization domain to be helical, hinting that there might be a break in the helicity of the oligomerization domain which confers a degree of flexibility to the HR-A and HR-B regions (Peteranderl et al., 1999). In tomato class A Hsfs the increased structural distance between the HR-A and HR-B regions, given by the typical insertion of 21 amino acid residues in the linker region, might further promote higher degree of flexibility to

the coiled-coil of the HR-B region to form several protein interactions besides those that ensure homotrimer formation (see below).

3.1.8.2 The HR-B region is an interacting surface differently involved for interactions between class A Hsfs

The essential role of the HR-B region in the protein interactions of class A Hsfs is shown by site directed mutagenesis of its highly conserved hydrophobic amino acid residues (MM>AA, FL>AA and IF>AA respectively at positions 193/194, or 196/197, or 200/201). Indeed the two-hybrid assay experiments show that all these amino acid residues are essential for the homodimeric complex formation of HsfA2, since each double alanine mutation is sufficient to disrupt the interaction (Fig. 3.9). The possible significance of these hydrophobic residues in the specificity of the helix interface is revealed by the schematic helical wheel representation of the oligomerization domain of HsfA2 (Figure 3.8 B). Only in the case of a heptad repeat pattern led by the HR-A region (open circles) the performed mutations would localize and alter residues at position **a** and **d**, which are involved in stabilizing the helix interface and therefore they would critically compromise the homodimeric interaction of HsfA2. In addition Figure 3.8 B shows that the FL>AA mutation, the only one that affects the interaction between HsfA2 and HsfA1, also localizes at position **e**. Preponderance of hydrophobic residues at position **e** has been suggested to provide a suitable surface for protein interaction as well as to cause aggregation (Peteranderl et al., 1999; Shu et al., 1999). Considering the large number of conserved hydrophobic residues at position **e**, it seems possible that a different helical interface is responsible for the heterodimeric interaction of HsfA2 with HsfA1. It therefore appears that the steric characteristics of the hydrophobic residues in the HR-B region of HsfA2 are differentially involved in the contact interface between the proteins and it suggests that the helix interface for homo- and hetero-complex formation might be different. However, the contradictory results about HsfA2 and HsfA3 interaction (Fig. 3.9) do not agree with this hypothesis; nevertheless, they suggest that other amino acid sequences, outside the linker and HR-B region, are responsible for conferring specificity to

the heterodimeric interaction. A most plausible domain would be the HR-A region. This hypothesis is in agreement with the data presented in Fig. 3.9, where mutated HsfA2 and HsfA3 proteins can interact only when the HR-A region is present (compare samples 7, 11, 15 in B to samples 12, 13, 14 in A). This would correlate with studies about the trimerization domain of *K. lactis*, which indicate that both HR-A and HR-B regions are critical in stabilizing the interaction between coiled-coil structures (Peteranderl et al., 1999).

3.1.9 Functional cooperation of Hsfs at DNA level

As previously mentioned (3.1.5), formation of stable and specific tomato Hsf homo- or heterocomplexes might not decisively influence the transcriptional activities of Hsf-DNA complexes, but rather the bindings of the individual proteins to the HSE sites.

The binding of Hsfs to DNA has been shown to be highly cooperative (Topol et al., 1985; Xiao et al., 1991; Wang et al., 1994; Bonner et al., 1994). Indeed recent studies have shown that cooperative interactions exist both between subunits within a Hsf trimer and between adjacent trimers (Littlefield and Nelson, 1999 and references therein).The parts of the protein critical for these cooperative DNA-binding activities have been found to be the DNA-binding and trimerization domains (Littlefield and Nelson, 1999 and references therein).

Functional cooperation exists between Hsf homotrimers and also between different Hsfs at DNA level displaying diverse effects. For example, in both yeast and *Drosophila*, cooperative interactions between Hsf homotrimers have been shown to be either important (yeast) or critical (*Drosophila*) for normal heat stress-inducible transcription (Cohen and Meselson, 1988; Amin et al., 1994; Bonner et al., 1994). Functional cooperation between human Hsf1 and Hsf2, in human erythroleukemia cells following induction by hemin and heat stress treatment, synergistically induce *hsp70* gene transcription (Sistonen et al., 1994).

Interestingly, direct protein interactions of Hsf with other transcription factors (excluding general components of the transcriptional machinery) have been sometimes demonstrated to be functionally relevant at promoters of heat stress

genes. That is the case of human Hsf1 and STAT-1 (Stephanou et al., 1999), chicken HsfA3 and c-Myb (Kanei-Ishii et al., 1997) and tomato HsfA1 and ABI3 (Rojas et al., 1999).

These data indicate that future studies investigating the nature of tomato Hsf-DNA interactions at promoters with diverse HSEs architecture are needed to establish whether cooperative binding exists and to what extent cooperative binding and functional synergy correlate with the overall transactivation potential of homo- and heterocomplexes of tomato Hsfs.

3.2 Protein interactions between HsfA2 and small heat stress proteins

Many aspects of expression and intracellular localization of the heat stress inducible transcription factor HsfA2 are very similar to those of cytoplasmic small heat stress proteins (sHsps). Immunofluorescence, immunoelectron microscopy and ultracentrifugation analysis well document characteristic changes of the intracellular localization of HsfA2 and sHsps. For example, after 2 h of heat stress with ongoing protein synthesis, a considerable amount of HsfA2, together with sHsps, can be detected in large cytoplasmic aggregates, the heat stress granules (HSGs), and later on, at the end of a 2 h recovery period, most of the HsfA2 is found in soluble form in the cytoplasm (Scharf et al., 1998b).

The first evidence for a direct physical interaction between the HsfA2 and the sHsps was found in a two-hybrid screening for HsfA2 interacting proteins, that resulted in the identification of several clones for the tomato class II sHsp (Chen A., diploma work, 1999). Interestingly no members of class I sHsp could be identified in this screening. These observations led to the following experiments with pull-down and yeast two-hybrid assays aimed at the further characterization of the interaction between HsfA2 and the small heat stress proteins.

3.2.1 *In vitro* interaction between HsfA2 and cytoplasmic sHsps

In order to investigate whether HsfA2 could interact with any cytoplasmic sHsp, several sHsps were used in the following pull-down assays. sHsp cDNAs were expressed as GST-tagged proteins in *E. coli*: class I Lp-Hsp17.6 (aa 1-154), class II Lp-Hsp17 (aa 1-155), class IIs Lp-Hsp17 (aa 7-143), class II Ps-Hsp17.7 (aa 2-157) and class II At-Hsp17.6 (aa 1-155). Expression and purification of the recombinant proteins and generation of the protein affinity-resins were performed according to standard protocols as well as the production of whole cell extracts from yeast strains expressing wild type or mutated forms of HsfA2 (see Materials and methods and 3.1.1).

To examine whether HsfA2 could interact equally with the tomato cytoplasmic sHsps so far identified, GST Lp-sHsp resins were incubated with whole cell extract from a yeast strain expressing HsfA2. Figure 3.12 B shows that HsfA2 interacts efficiently with immobilized GST Lp-Hsp17(II), weakly with immobilized GST Lp-Hsp17(IIs) and not at all with immobilized GST Lp-Hsp17.6(I) (lanes 5, 4 and 3). To investigate if structural domains of HsfA2 are required for determining or stabilizing this interaction a different pull-down assay was carried out using whole cell extract from yeast strains expressing partially deleted forms of HsfA2 (see scheme in Fig. 3.12 A). Evidently the oligomerization domain of HsfA2 seems to play a major role in the protein interaction; Figure 3.12 C shows that the C-terminal deleted protein HsfA2ΔC300 (lane 4) can be specifically retained by the GST Lp-Hsp17(II) resin whereas HsfA2Δ7/8,ΔC323, presenting also a deletion in the oligomerization domain, is hardly retained (lane 6). As negative control GST-resin was also incubated with each yeast whole cell extract to exclude any non-specific binding (lane 2 in B, lanes 3 and 5 in C). Since amino acid sequence comparisons have revealed that both class I and class II sHsps from any species are more related to proteins of the same class from a different species than to each other (Waters et al., 1996; Löw et al., 2000), we investigated if HsfA2 could interact with class II sHsps from pea (*Pisum sativum*) and from *Arabidopsis thaliana*. Figure 3.13 shows that in contrast to the efficient and specific interaction of HsfA2 with class II Hsp17 from tomato (lane 3), a comparable interaction with

the homologous class II proteins from pea (lane 4) and *Arabidopsis* (lane 5) is not detectable.

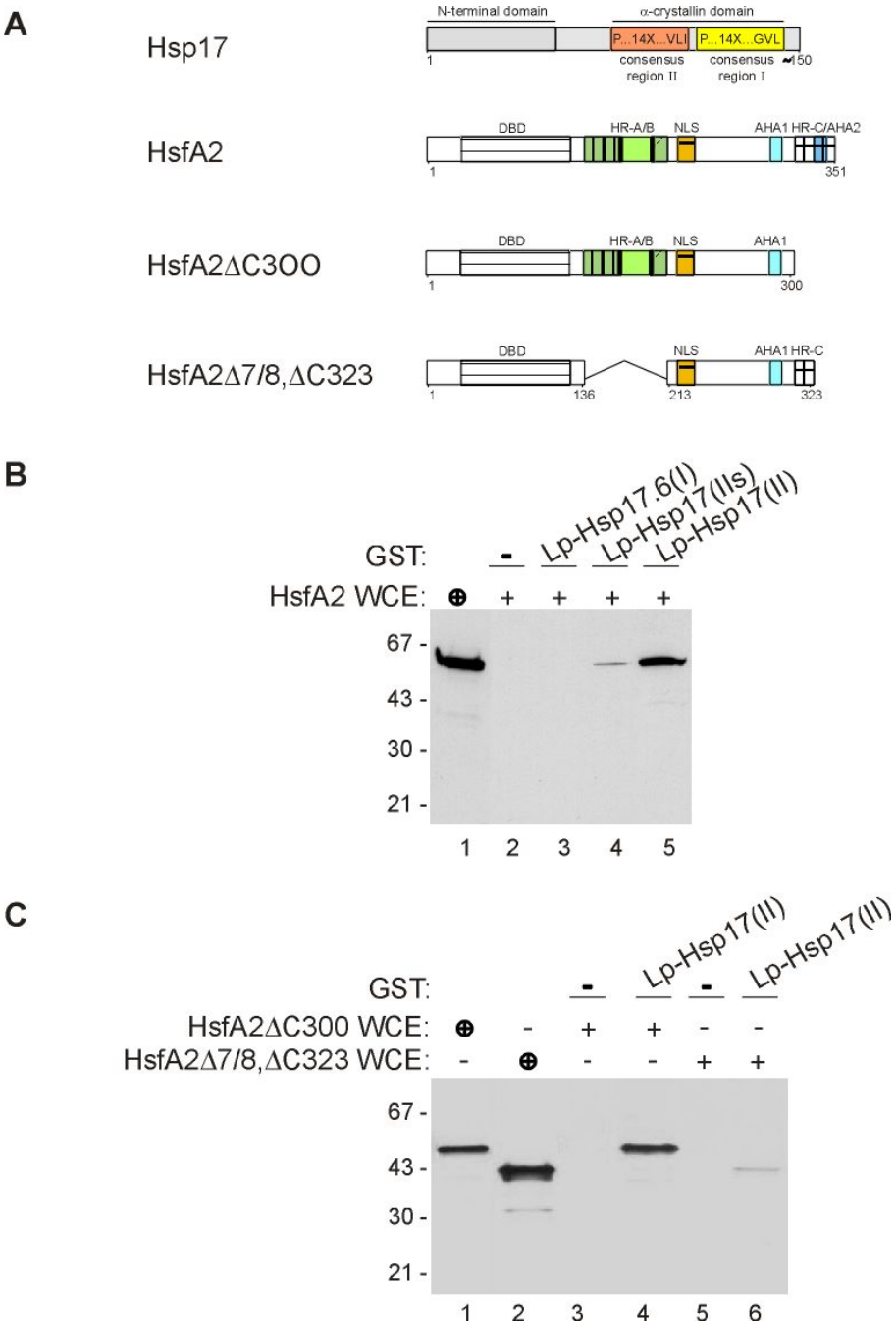


Figure 3.12 Interactions between HsfA2 and tomato sHsps (pull-down assay). (**A**) Schematic representation of cytoplasmic Hsp17, HsfA2 and its mutated forms indicating functional modules. (**B**) HsfA2 strongly interacts with Lp-Hsp17(II). 450 µg of yeast whole cell extract (WCE) containing HsfA2 were incubated with 5 µg of GST (lane 2), GST Lp-Hsp17(I) (lane 3), GST Lp-Hsp17(IIs) (lane 4) and GST Lp-Hsp17(II) (lane 5) resins. (**C**) Interaction between deleted forms of HsfA2 and Lp-Hsp17(II). 450 µg of whole cell extract containing HsfA2ΔC300 or HsfA2Δ7/8,ΔC323 were incubated with 5 µg of GST (lanes 3 and 5) or GST Lp-Hsp17(II) (lanes 4 and 6) resin. Half amount of the eluted samples was analyzed by SDS-PAGE and Western blotting with anti-HsfA2 antibodies (pep6 in **B** and HsfA2HN in **C** respectively). ⊕ indicates the amount of HsfA2 proteins in 10 µg of the corresponding yeast whole cell extract (lane 1 in **B**, and lanes 1 and 2 in **C**). Molecular masses (in kDa) of markers are indicated on the left.

A

```
                                                                                    
Lp-Hsp17  (II)     1- MDLRLMGIDNTPLFHTLQHMMEAAGEDS----VNAPSKKYVRDAKAMAATPVDVKEYPD
Ps-Hsp17.7(II)     1- MDFRLMDLD-SPLFNTLHHIMDLTDDTT-EKNLNAPTRTYVRDAKAMAATPADVKEHPN
At-Hsp17.6(II)     1- -----MDLGRFPIISILEDMLEVPEDHNNEKTRNNPSRVYMRDAKAMAATPADVIEHPN
                          *.:.  *::  *.:::: . : .     * *:: *:**********.** *:*:

                                                      α-crystallin domain
Lp-Hsp17  (II)       SYVFVVDMPGLKSGDINVQVEEDNVLLISGERKRE-EEKEGVKFIRMERRVGKFMRKFSL
Ps-Hsp17.7(II)       SYVFMVDMPGVKSGDIKVQVEDENVLLISGERKRE-EEKEGVKYLKMERRIGKLMRKFVL
At-Hsp17.6(II)       AYAFVVDMPGIKGDEIKVQVENDNVLVVSGERQRENKENEGVKYVRMERRMGKFMRKFQL
                     :*.*:*****:*..:*:****::***::****:** :*:****:::****:**:**** *
                              Consensus region II

Lp-Hsp17  (II)       PENANTDAISAVCQDGVLTVTVRKLPPPEPKKPKTIQVKVA -155
Ps-Hsp17.7(II)       PENANIEAISAISQDGVLTVTVNKLPPPEPKKPKTIQVKVA -157
At-Hsp17.6(II)       PENADLDKISAVCHDGVLKVTVQKLPPPEPKKPKTIQVQVA -155
                     ****: : ***:.:****.***.***************:**
                       Consensus region I
```

B

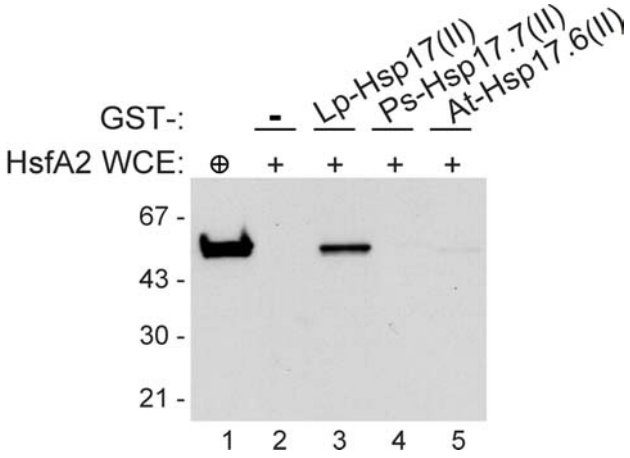


Figure 3.13 Interactions between HsfA2 and heterologous cytoplasmic sHsps of class II (pull-down assay). (**A**) Multiple sequence alignment of class II small heat stress proteins from tomato (Lp-Hsp17(II)), pea (Ps-Hsp17.7(II)) and *Arabidopsis* (At-Hsp17.6(II)); numbers denote the first and the last amino acid residues of each protein. The α-crystallin domain and the consensus region II and I are overlined in black and underline d in red respectively; important residues are labeled in red. Sequences were aligned with the Clustal W multiple sequence comparison program: '*****' indicates fully conserved residue; '**:**' indicates high similarity and '**.**' indicates low similarity of residues. (**B**) HsfA2 can interact with Hsp17(II) from tomato (lane 3) but not from pea (lane 4) or *Arabidopsis* (lane 5). Western analysis was performed with anti-HsfA2 antibody (pep6). See legend to Figure 3.12 for further explanations.

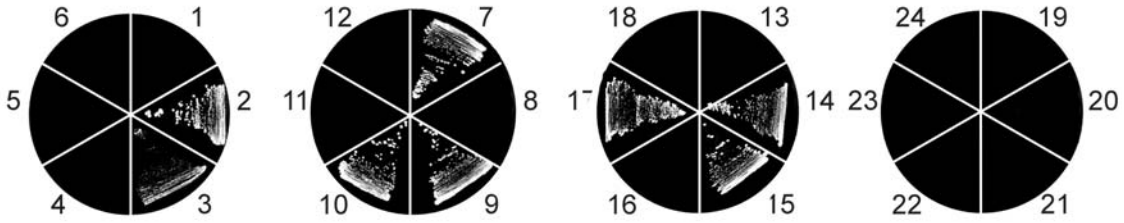
3.2.2 *In vivo* interaction between HsfA2 and cytoplasmic sHsps

The yeast two-hybrid assay was performed in order to confirm the physical interaction between HsfA2 and the small heat stress proteins *in vivo*. Moreover the interaction between sHsps was also tested as control and comparison to HsfA2 and sHsps protein interactions. Features of HsfA2 bait and other sHsps baits and preys are listed below in Table 3.2.

Figure 3.14 shows that HsfA2 can strongly interact with Lp-Hsp17(II) (sample 2), weakly with Lp-Hsp17(IIs) (sample 3) and not at all with Lp-Hsp17.6(I) (sample 1) or with sHsps from pea (samples 4 and 5). These data confirm the effects observed in the pull down assays (Fig. 3.12 and Fig. 3.13). In addition, protein interaction between sHsps is class-specific: Lp-Hsp17.6 (I) can interact with itself (sample 7) and with class I Ps-Hsp18.1 (sample 10), but not with class II sHsps (samples 8 and 11). On the other hand Lp-Hsp17(II) can interact with itself (sample 14) and with class II Ps-Hsp17.7 (sample 17), but not with the class I sHsps (samples 13 and 16). Interestingly, both class I and class II cytoplasmic sHsps can interact with Lp-Hsp17(IIs) (samples 9 and 15).

	Name	**Coding sequence**	**Code N.**
Baits:	HsfA2	aa 98-351:W297>A, W337>E,and L341>A	RL130
	Lp-Hsp17.6(I)	aa 1-154	MK49
	Lp-sHsp17(II)	aa 1-155	MK50
Preys:	Lp-sHsp17.6(I)	aa 1-154	MK48
	Lp-sHsp17(II)	aa 1-155	AC76.1
	Lp-sHsp17(IIs)	aa 7-143	MP8.2
	Ps-sHsp18.1(I)	aa 1-153	MK45
	Ps-sHsp17.7(II)	aa 1-158	MK40

Table 3.2 Baits and preys used for testing HsfA2 and sHsps protein interactions in the two-hybrid assay. Characteristics of HsfA2 bait and other general details have been previously described (see 3.1.4). Name and coding sequence of proteins as well as code number of plasmids are shown. See Appendix C (Plasmid Maps) for further details.



Bait \ Prey	Lp-Hsp17.6(I)	Lp-Hsp17(II)	Lp-Hsp17(IIs)	Ps-Hsp18.1(I)	Ps-Hsp17.7(II)	GaL4 AD
HsfA2	1	2	3	4	5	6
Lp-Hsp17.6(I)	7	8	9	10	11	12
Lp-Hsp17(II)	13	14	15	16	17	18
Gal4 BD	19	20	21	22	23	24

Figure 3.14 Interactions between HsfA2 and cytoplasmic sHsps (two-hybrid assay). The indicated proteins, fused to the yeast Gal4 DNA-binding domain (aa 1-147, Gal4 BD; bait) or to the yeast Gal4 activation domain (aa 768-881, GAL4 AD; prey) were coexpressed in the yeast strain YRG-2 according to the table. See legend to Figure 3.6 for further explanations.

3.2.3 HsfA2 interacts specifically with class II sHsp from tomato

Recent work about the characterization of tomato class I and class II sHsps has shown that their genomic organization, transcription, protein expression and *in vivo* chaperone activity are very similar despite differences in their amino acid sequences (Löw et al., 2000). However these investigations could not suggest any functional role and biological reason why two well defined and differentiated classes of sHsps are present in the plant cytoplasm.

Interestingly the *in vitro* and *in vivo* protein interaction studies between HsfA2 and sHsps give the first evidence for a possible difference (see 3.2.1 and 3.2.2). Indeed HsfA2 specifically interacts with class II, weakly with class IIs and not with class I tomato sHsps indicating that the protein interaction is class-specific. Moreover, HsfA2 can not interact with other class II small heat stress proteins from pea or *Arabidopsis* suggesting that the protein interaction is also species-specific. These results, taken together, suggest that HsfA2 interacts

specifically with tomato sHsp(II). In addition, this interaction is mediated by the oligomerization domain of HsfA2 and other class A Hsfs do not interact with tomato sHsp(II) (data not shown). Therefore, the specificity of the interaction between HsfA2 and class II sHsp from tomato indicates that each cytoplasmic sHsp might be specialized to bind and recognize a specific protein.

Several factors like low amino acid sequence homology between sHsps (Waters et al., 1996; Löw et al., 2000) and the considerable variation in size and subunit composition of the sHsp oligomers, which have been reported according to the class as well as to the species (Chen et al., 1994; Lee et al., 1995; Jinn et al., 1995; Helm et al., 1997; Kirschner et al., 2000), might explain the different protein interactions observed between HsfA2 and cytoplasmic sHsps and contribute to the specific interaction of tomato sHsp(II) with HsfA2.

Plant sHsps, as well as all the other members of the Hsp20 chaperone family, are characterized by a significantly reduced homology in the N-terminal domain and C-terminal tail that flanks a sequence of about 100 residues, which is homologouos to the αB-crystallin from the vertebrate eye lens and thus is called α-crystallin domain (Klemenz et al., 1991; Waters et al., 1996).

Amino acid sequence alignment of tomato cytoplasmic sHsps used during this study is presented in Figure 3.15: the high identity is mainly limited to residues of the two subdomains of the α-crystallin domain: the residues P-$X_{(14)}$-X-V/L/I-V/L/I within consensus region II and the residues P-$X_{(14)}$-G-V-L within consensus region I (Waters et al., 1996, Löw et al., 2000).

The interaction surface of the α-crystallin domain permits a stable and class specific interaction between sHsps (Kim et al., 1998; Berengian et al., 1999; Kirschner et al., 2000), but it is not sufficient to mediate the interaction with HsfA2 (Fig. 3.14). Indirectly these results also suggest that the N-terminal domain of the class II sHsp might have a central role in the interaction with HsfA2. As can be observed in Figures 3.13 A and 3.15 the N-terminal domains of the cytoplasmic sHsps have a significantly reduced homology; this divergence is observed in all members of plant sHsps. Indeed, amino-terminal consensus sequences, which are unique to each class, have been characterized (Waters et al., 1996) and it has been suggested that the N-

terminal domains of the proteins serve important functional role for substrate binding (Vierling, 1991; Kim et al., 1998; Löw et al., 2000).

Recent cryo-electron microscopy studies have shown that the sHsp oligomeric complexes display a remarkable structural flexibility in solution; that is even valid for the most symmetrical sHsp examined, Hsp16.5 from *Methanococcus jannaschii* (Haley et al., 2000). These results indicate that structural variations in the N-terminal domain and C-terminal tail, as well as subtle differences in the α-crystallin domain fold, may be responsible for the dramatic differences observed in the quaternary structure of the sHsps. Since

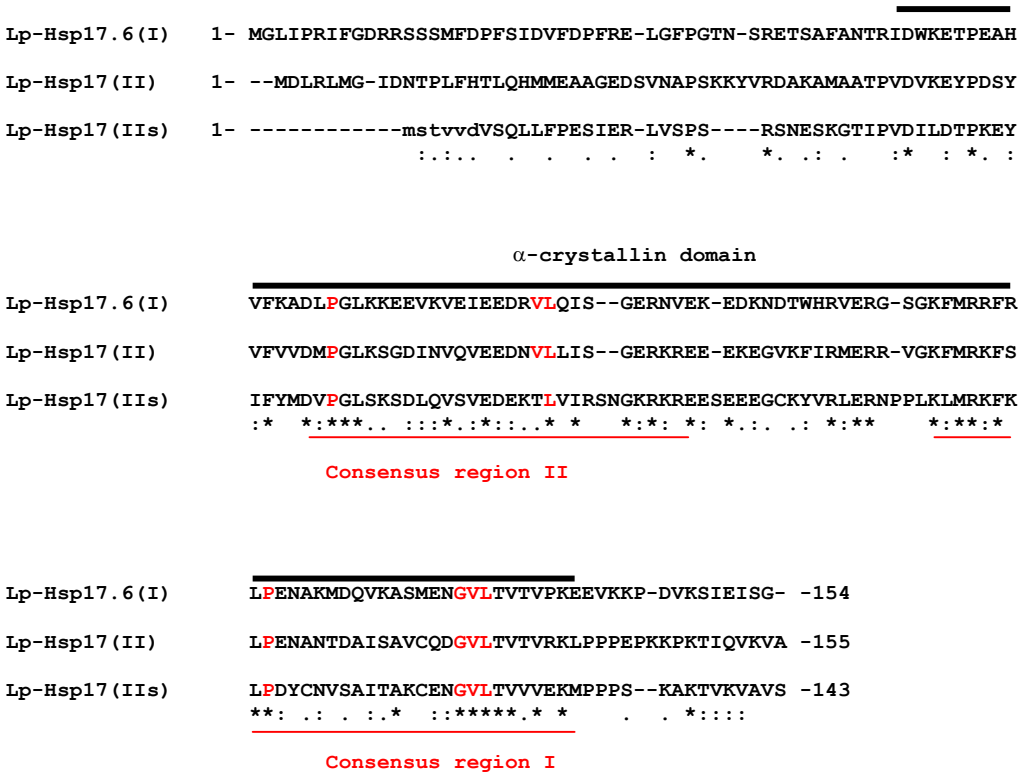


Figure 3.15 Amino acid sequence alignments of tomato cytoplasmic sHsps. Protein sequences are labeled with the protein name to the left: numbers denote the first and the last amino acid residues. Sequences were aligned with the Clustal W multiple sequence comparison program; "**-**" indicates a gap introduced to optimize the alignment. The α-crystallin domain and the consensus region II and I are respectively overlined in black and underlined in red; important residues are labeled in red. Note that Lp-Hsp17(IIs) sequence is incomplete: the cDNA was truncated and did not include sequences encoding the amino-terminal domain; small letters indicate the amino acid residues predicted from the 5` cDNA sequence recently identified by 5`RACE method, but not present in the Lp-Hsp17(IIs) vectors used in this study.

one of the function of sHsps is to bind partially denatured polypeptides in order to preserve their folding competent state during stress conditions, the suggestion is that the dimension and plasticity of the quaternary structure may be of functional importance for specific recognition and binding of defined proteins, e.g. HsfA2.

3.2.4 HsfA2 and the tomato cytoplasmic small heat stress proteins with respect to the HSG complexes

The heat stress dependent and reversible association of HsfA2 with the plant-specific and cytoplasmic HSG complexes (Scharf et al., 1998b) as well as its physical interaction with class II small heat stress proteins (3.2.1 and 3.2.2) are properties not found for any other tomato Hsfs (data not shown; Bharti et al., 2000).

Formation of HSGs is a general phenomenon in all plant tissues expressing high levels of sHsp synthesis; they form under heat stress and decompose upon recovery (Nover et al., 1983; Neumann et al., 1984; Nover et al., 1989a). The HSGs are composed predominantly of cytoplasmic sHsps; however, Hsp70, ribonucleoproteins and partially denatured proteins have also been described as components (Nover et al., 1983 and 1989b; Neumann et al., 1984; Scharf et al., 1998b; Smỳkal et al., 2000). Interestingly recent work with isolated and purified HSG complexes from tobacco cell suspension has demonstrated that they display a chaperone-like activity (Smỳkal et al. 2000).

Multiple studies have shown that during high-temperature stress sHsps prevent irreversible protein aggregation by binding unfolded or misfolded proteins in an ATP-independent manner and maintaining them in a state competent for refolding by ATP-dependent chaperones (e.g. Hsp70 and Hsp90). Under more physiological conditions the Hsp70 chaperone system facilitates refolding and ensures the final protein native state (reviewed by Forreiter and Nover, 1998; Lee and Vierling, 2000). The functional cooperation between sHsps and the Hsp70 systems has been suggested by several experiments *in vitro* and *in vivo* (Ehrnsperger et al., 1997; Lee at al., 1997; Forreiter et al., 1997; Lee and Vierling, 2000; Löw et al., 2000) and a general

model for their molecular chaperone activity is presented in Figure 3.16. In addition there is evidence that the Hsp70 system does not substitute for sHsp activity in maintaining folding competence and protein solubility and that no high specific and direct interactions of sHsps themselves with the folding system components are required (Lee and Vierling, 2000).

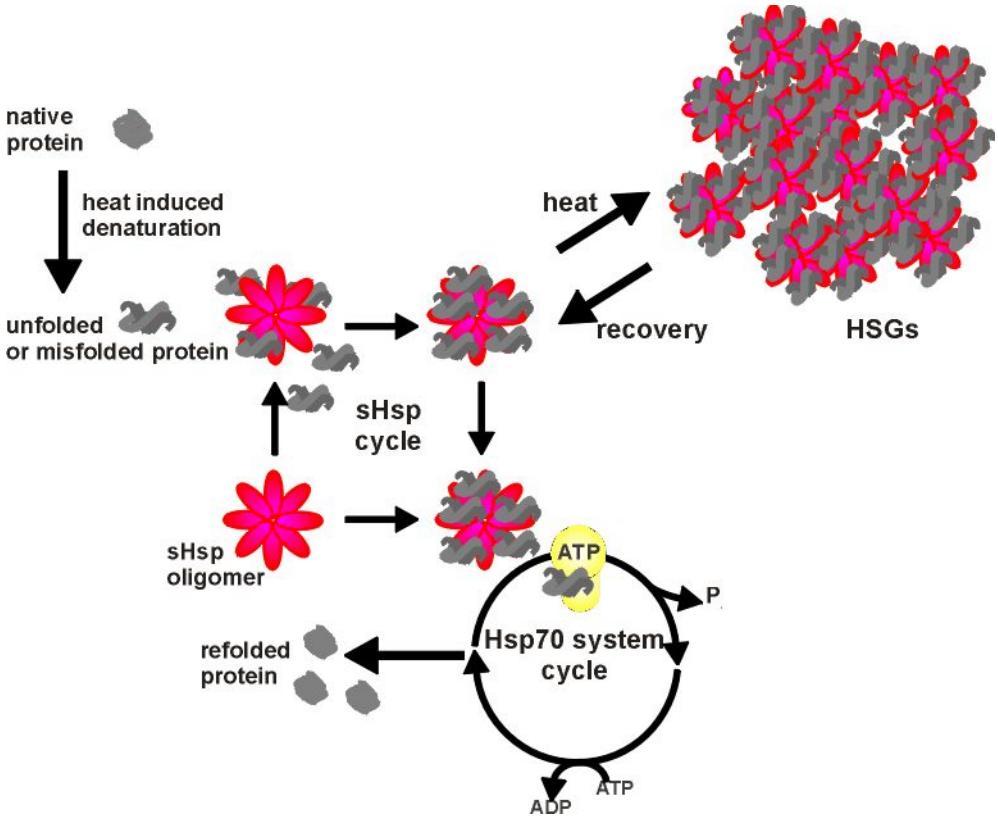


Figure 3.16 Model for the molecular chaperone activity of sHsps/Hsp70 system. Under stress conditions unfolded/misfolded proteins accumulate in the cytoplasm and are bound by cytoplasmic sHsp oligomeres, which keep them in a competent refolding state. This allows them to undergo multiple rounds of interaction with the Hsp70 system (Hsp70 and co-chaperones) for ATP-dependent folding and to achieve their final native state. In plant cells under prolonged and severe heat stress both class I and class II sHsp oligomers form cytoplasmic aggregates, the heat stress granules (HSGs), which store the partially unfolded proteins. Under recovery conditions these complexes disassemble releasing the sHsp oligomeres, which can interact with the Hsp70 system for refolding. (modified from Forreiter and Nover, 1998).

It has been reported that the HSGs might function as sites for transient storage and protection of proteins during severe heat stress conditions when the action and availability of energy-dependent chaperones might be limited (Forreiter and Nover, 1998; Löw et al., 2000). This would suggest that the specific interaction of tomato class II sHsp with HsfA2 may protect selectively the latter from denaturation and/or aggregation and facilitate its localization in the HSGs, where sHsps and Hsp70 chaperone systems can act in concert and in spatial and functional proximity, that is also time and heat stress dependent.

Recent findings in our group suggest that HsfA2 and tomato sHsp(II) interaction as well as HsfA2 localization in the HSGs might also correlate to a fine regulation of the plant heat stress response.

- Analyses of transgenic tomato plants with increased or decreased levels of tomato Hsfs suggest that newly synthesized HsfA2 protein induces transcription of sHsps (Mishra S.-K., diploma work, 2000).
- Analyses in tobacco protoplast assays indicate that HsfA2 induces expression of endogenous sHsps (Scharf K.-D., unpublished results).
- In other tobacco protoplast reporter assays the coexpression of HsfA2 and tomato sHsp(II), but not tomato sHsp(I) or other sHsps, reduces the activation of a Hsf-dependent reporter gene (Port M., unpublished results from our group). This suggests that the specific interaction between HsfA2 and sHsp(II) interferes with the activator function of HsfA2.

All these findings and observations agree with the hypothesis that tomato sHsp(II) is a negative regulator of HsfA2 activity. Under heat stress conditions nuclear localization of HsfA2 might lead to transcription of sHsps, which are involved in maintaining folding competence and solubility of proteins in the cytoplasm. However, during a prolonged and severe heat stress response, expression of sHsps might be higher than necessary and their transcription should consequently be turned off. To achieve that, sHps(II), whose concentration is increased in the cytoplasm, might bind HsfA2 and localize it in the HSGs. This event leads to turning off sHsps transcription and participate in the down regulation of the heat stress response.

3.3 Identification of putative HsfB1 interacting proteins

3.3.1 Searching for HsfB1 interacting proteins

Although protein interactions are often amenable to analysis in the yeast two-hybrid system, it is sometimes preferable to study these interactions in a plant environment. The formation of a protein complex may be subjected to regulatory influences not faithfully represented in yeast. Moreover, in tomato cell culture, HsfB1 is expressed under heat stress conditions and coexists with other tomato Hsfs and other heat stress proteins (see Fig. 1.6). These conditions might influence the stability or type of HsfB1 protein interactions and therefore its functions.

The yeast two-hybrid system proved not to be a suitable method to screen for proteins interacting with HsfB1 and, as an alternative, a protein-affinity chromatography approach (pull-down assay) was used. Several protein-affinity resins were necessary to characterize the protein/s interacting with HsfB1 and therefore the following fusion proteins, His_6-tagged at the C-terminus, were expressed in *Escherichia coli* BL21(DE3)pLysS strain:

- His_6-HsfB1, which represents almost the full length HsfB1 (aa 1-293);
- His_6-HsfB1 DBD, which represents the DNA-binding domain of HsfB1 (aa 1-106);
- His_6-HsfB1 CTD, which represents the C-terminal domain of HsfB1 (aa 140-293);
- His_6-HsfA1, which represents almost the full length HsfA1 (aa 23-527), was used as a control, that means as a protein belonging to the Hsf family but with different structural and functional characteristics as compared to HsfB1.

Expression and purification of the recombinant proteins were performed according to standard protocols described in Materials and methods. The purified proteins were used to saturate the Ni-NTA resin via the His_6-tag. The quality of the Hsf-resins was analyzed by SDS-PAGE and Coomassie staining

and a representative analysis is shown in Figure 3.17. Proteins have molecular weights of approx. 57 kDa for His_6-HsfA1, 35 kDa for His_6-HsfB1, 20 kDa for the C-terminal domain (His_6-HsfB1 CTD) and 15 kDa for the DNA-binding domain (His_6-HsfB1 DBD) of HsfB1. Partial protein degradation, representing minor contaminants, was only present in the HsfA1-resin (lane 1).

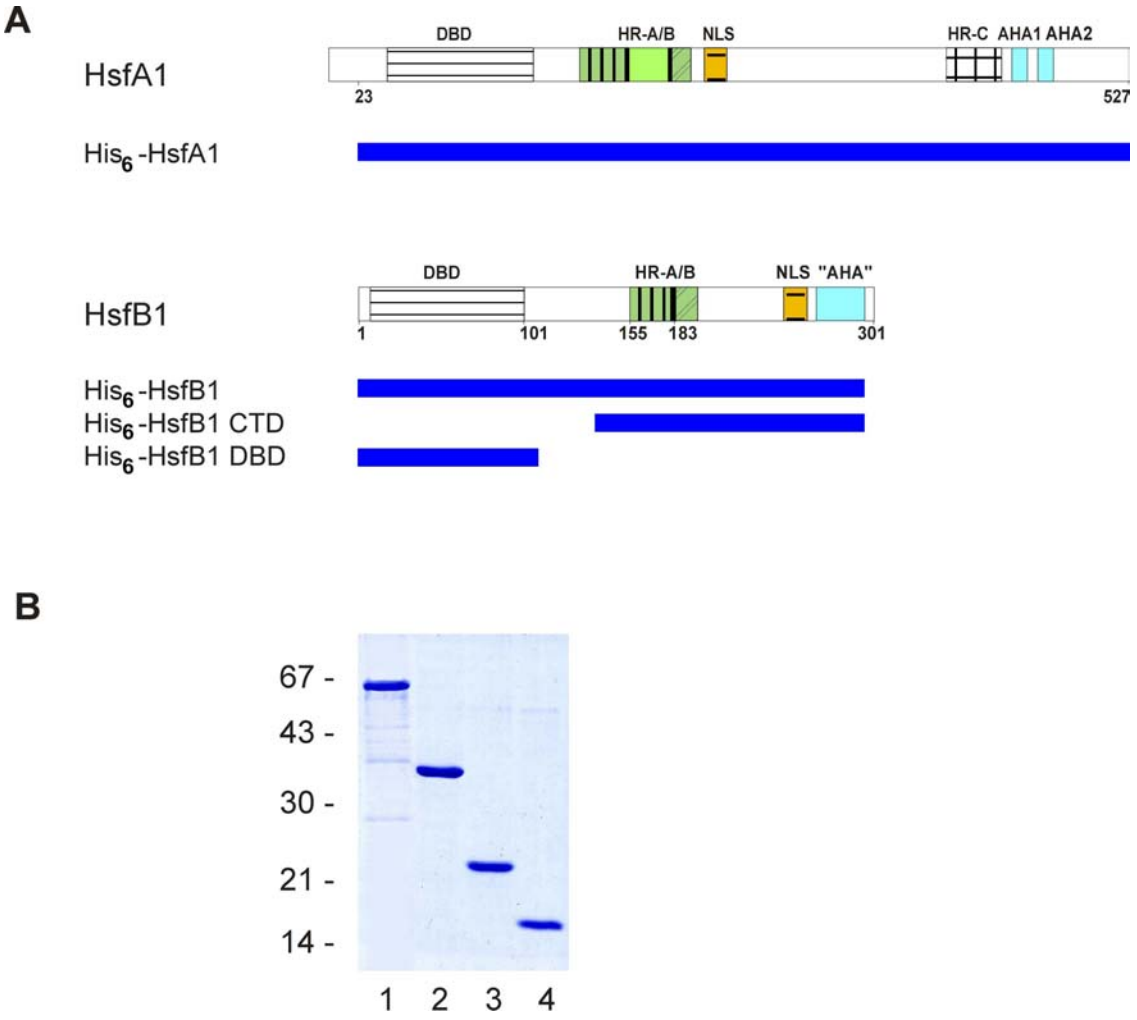


Figure 3.17 Hsf affinity resins. (**A**) Schematic representation of the His_6-Hsf fusion proteins: block diagrams indicate presence and position of the different functional domains; see Introduction and Fig. 1.3 for further explanations about functional modules. (**B**) 5 µg of each Hsf-resin is visualized by Coomassie staining on a 12% SDS-PAGE gel: His_6-HsfA1 (aa 23- 527; lane 1), His_6-HsfB1 (aa 1-293; lane 2), C-terminal domain (His_6-HsfB1 CTD, aa 140-293; lane 3) and DNA-binding domain (His_6-HsfB1 DBD, aa 1-106; lane 4) of HsfB1. Molecular masses (in kDa) of markers are indicated on the left.

In general, for each pull-down assay the following reactions were set up to evaluate which proteins were specifically associated with the Hsf-resin and therefore interacting with Hsf:

- Hsf-resin alone: to monitor the integrity of the resin (after the binding-reaction time);
- Hsf-resin with whole cell extract from tomato cell culture: to asses which proteins were specifically retained by the Hsf fusion protein;
- Ni-NTA resin with whole cell extract from tomato cell culture: to asses which proteins were non-specifically retained.

Samples were incubated and gently mixed; after extensive washing steps, three different elutions in series were used to validate the protein-protein interactions; the sequential elutions were by:

- high salt-elution (High Salt): presence of 1M NaCl should ensure elution of proteins that interact mainly through strong ionic interactions;
- imidazole-elution (Imidazole): presence of imidazole should elute by competition the His_6-tag fusion protein, that saturates the resin, and those proteins that are strongly associated with it;
- SDS-elution (SDS): presence of SDS should ensure a complete elution of any residual protein in the resin; this step is a general control for the efficiency of the two previous elutions.

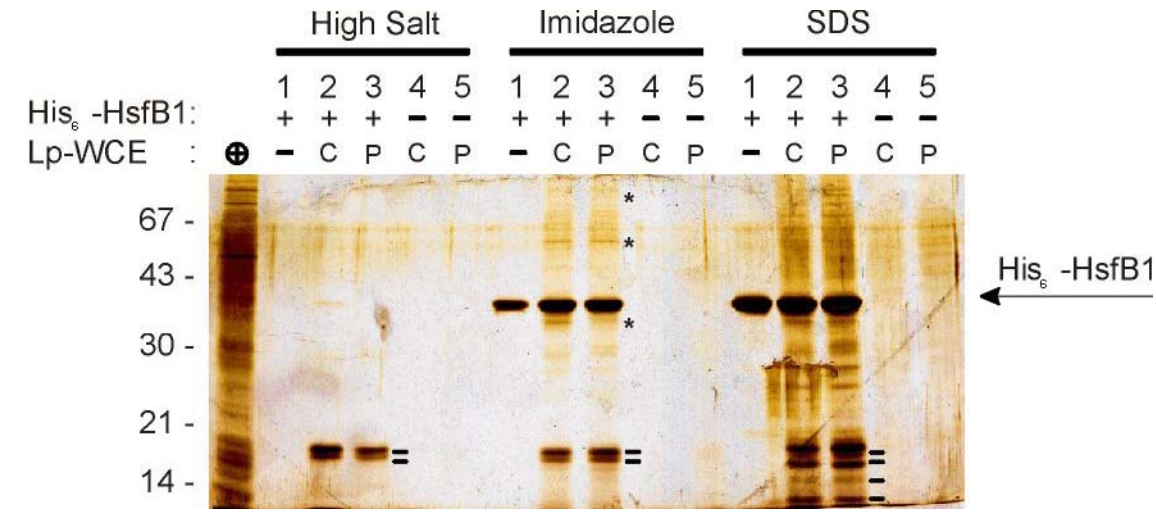
The eluted samples were separated by SDS-PAGE and visualized by silver staining. A careful comparison between samples was made to identify putative interacting proteins for HsfB1.

3.3.1.1 Partial purification of HsfB1 interacting proteins

As a first step HsfB1-resin was incubated with whole cell extract prepared from tomato cell culture grown either at control conditions (C) or with a preinduced heat stress treatment (P) (see Material and methods). Figure 3.18 A shows that two polypeptides (black bars) with molecular masses ranging from ≥ 14 to 20 kDa are eluted under high salt conditions (samples 2 and 3 in High Salt). These two polypeptide masses are also present in the eluates of the other two subsequent elutions (samples 2 and 3 in Imidazole and in SDS). In the latter elution, two other smaller polypeptides (black bars), with molecular masses ranging from ≥ 10 to 14 kDa are also evidently present in samples containing HsfB1-resin (samples 2 and 3 in SDS). Larger proteins of apparent molecular masses of 32 kDa, 60 kDa and 90 kDa (indicated by asterisks) also copurified with His_6-HsfB1 in the Imidazole eluates beside of other weakly detectable polypeptides (samples 2 and 3 in Imidazole).

To find out whether the copurified proteins were specifically associating with HsfB1 or also with other members of the Hsf family, a similar experiment was performed using this time the HsfA1-resin. Figure 3.18 B shows that small polypeptides with molecular masses ranging from ≥ 10 to 20 kDa, as those (black bars) observed copurifying with His_6-HsfB1 in the previous experiment (Fig. 3.18 A) are not associating with His_6-HsfA1. In this experiment the integrity of the HsfA1-resin was affected, i.e. His_6-HsfA1 is partially eluted at high salt (samples 1, 2 and 3) and protein degradation is evident (samples 1 in Imidazole and in SDS). Some other proteins of molecular masses ranging from 32 to 80 kDa (indicated by asterisks in Fig. 3.18 B) are also observed together with other not well defined proteins (samples 2 and 3 in Imidazole).

A



B

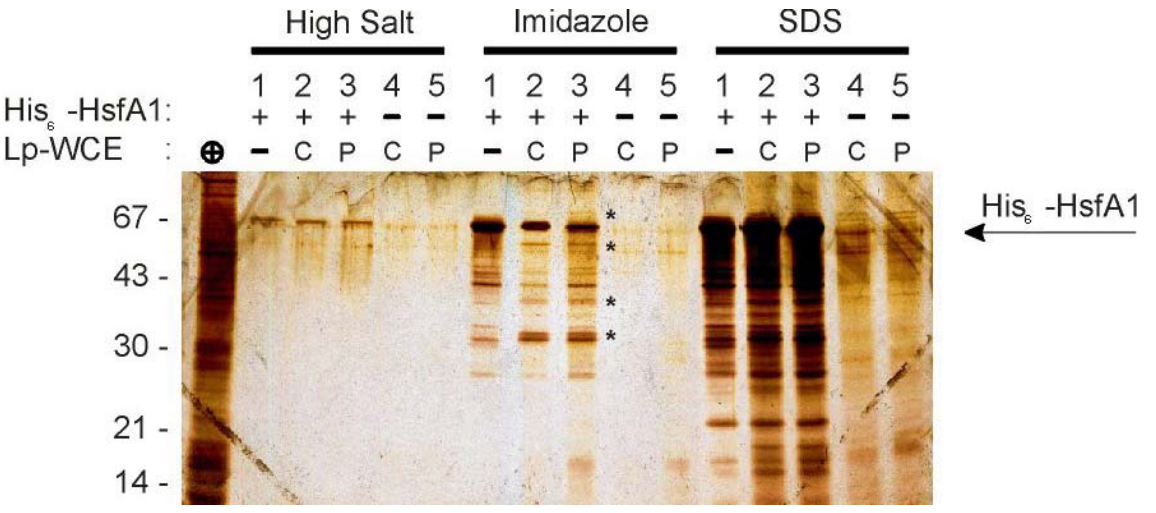


Figure 3.18 Partial purification of proteins interacting with His_6-HsfB1 (A) and with His_6-HsfA1 (B) (pull-down assays). The input and eluted fractions of the pull-down assays were separated on 12 % SDS-PAGE and visualized by silver staining. Samples were eluted stepwise by high salt-elution buffer (High Salt), imidazole-elution buffer (Imidazole) and finally by adding SDS-elution buffer (SDS). Samples and eluted fractions are indicated by the same numbers (1 to 5). On the left: the His_6-Hsf row indicates presence (+) or absence (-) of His_6-Hsf in the sample and the Lp-WCE row indicates presence of control (C) or preinduced heat stress (P) whole cell extract (WCE) from *Lycopersicon peruvianum* (Lp) cell culture or its absence (-) in the sample. The input (⊕) is 0.5 μg Lp-WCE from cell culture grown at control conditions. In the panel, the position of the main proteins (protein of interest)(**-**) and other proteins present in a lower amount and not further investigated (*****) are indicated; arrows point to the position of the His_6-Hsfs. Molecular masses (in kDa) of markers are indicated on the left.

3.3.1.2 Specificity of HsfB1 interacting proteins

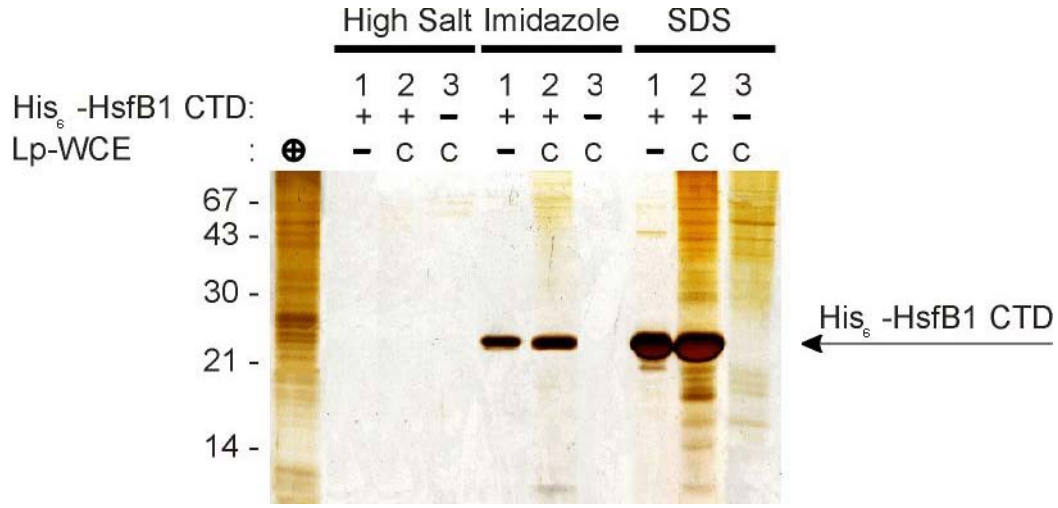
In order to verify whether a region or a specific structural domain of HsfB1, such as the oligomerization domain and the activation domain or the DNA-binding domain, was responsible for the protein interaction with the small proteins copurifying with HsfB1 (black bars in Fig. 3.18 A), two other pull-down assays were carried out.

HsfB1 CTD-resin, or HsfB1 DBD-resin, was incubated with whole cell extract of tomato cell culture grown only at control conditions (samples 2), since no difference was observed by using control or preinduced heat stress extracts in the previous experiment. The result is quite straightforward for the HsfB1 CTD pull-down since the elution profile is completely different from the one obtained with the HsfB1-resin (compare Fig. 3.18 A with Fig. 3.19 A): the small proteins, ranging in the size between 10 and 20 kDa, are not clearly identifiable and, moreover, no other relevant copurified proteins are visible.

The HsfB1 DBD pull-down assay (Fig. 3.19 B) results in an elution profile similar to the one presented in Figure 3.18 A, except that the integrity of the resin is already compromised by elution of the fusion protein at high salt (samples 1 and 2, in High Salt). Nevertheless, two polypeptides with molecular masses ranging from 14 to 20 kDa are copurifying and coeluting with HsfB1-DBD (black bars in samples 2). Two other smaller polypeptides, with molecular masses ranging from ≥ 10 to 14 kDa, are also present in the last elution (SDS).

These results indicate a specificity of the interaction between HsfB1 and the smaller polypeptides: no interaction is observed between the polypeptides and HsfA1 (Fig. 3.18 B), HsfA2 (data not shown) and the C-terminal domain of HsfB1 (Fig. 3.19 A). The DNA-binding domain of HsfB1 might be the interacting region (Fig. 3.19 B) although the interactions seem weaker when compared to results obtained with the full length protein (Fig. 3.18 A). This suggests that also the linker region connecting the DBD with the oligomerization domain of HsfB1 or the full length HsfB1 are required for effective strong protein interactions.

A



B

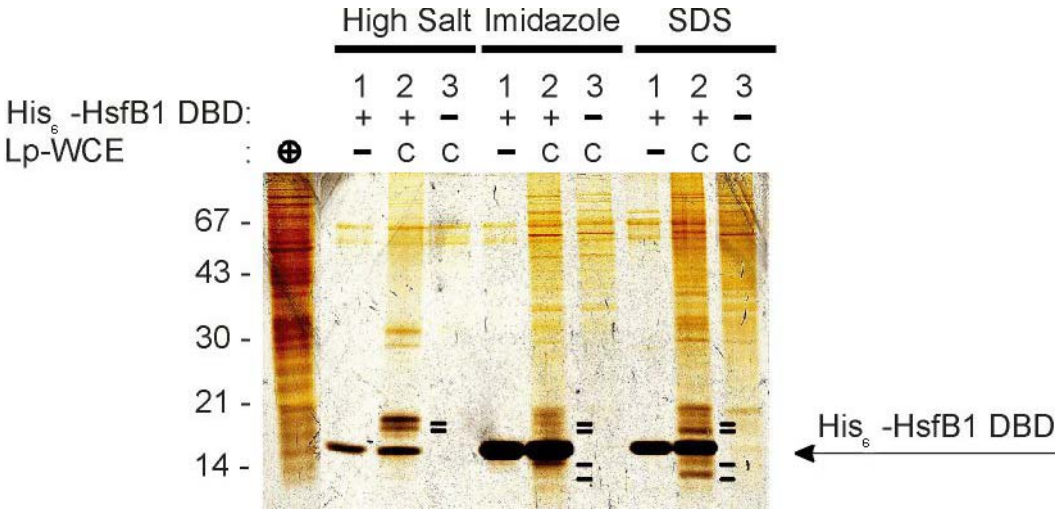


Figure 3.19 Partial purification of proteins interacting with His_6-HsfB1 CTD (A) or His_6-HsfB1 DBD (B) (pull-down assays). The input and eluted fractions of the pull-down assays were separated on 15 % SDS-PAGE and visualized by silver staining. See legend to Figure 3.18 for further explanations.

3.3.2 Identification of HsfB1 interacting proteins

The HsfB1 pull-down assay was carried out again, as described before, but scaled up to increase the yield of the small polypeptides with molecular masses ranging from 10 to 20 kDa that associate specifically with HsfB1. Eluted fractions (High Salt and Imidazole fractions) were dialyzed, pooled and separated on 15% SDS-PAGE. The bands corresponding to the polypeptides of interest (a, b, c and d in Fig. 3.20) were cut out from the gel after Coomassie staining and destaining (Fig. 3.20 B) to proceed for peptide mass fingerprinting analysis. Additionally, proteins were provided on a PVDF membrane after blotting (Fig. 3.20 C) for N-terminal sequencing analysis.

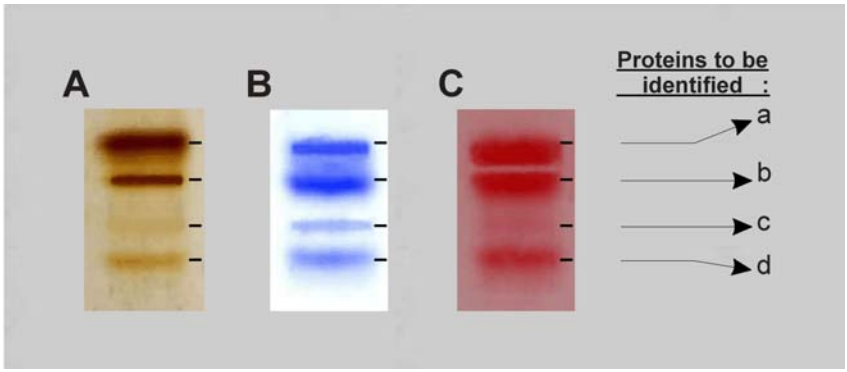


Figure 3.20 Major HsfB1 interacting proteins. The corresponding bands were visualized either by silver staining (**A**) as control or by Coomassie staining (**B**) before cutting out bands as gel slices and proceeding for peptide mass finger printing analysis. The corresponding region, after blotting on a PVDF membrane and Ponceau staining is shown in (**C**) before sending samples for N-terminal sequencing analysis.

3.3.2.1 Peptide mass fingerprinting analysis and amino acid composition identification

The Peptide mass fingerprinting analysis is a common protein identification technique that involves the generation of peptides from a protein using residue-specific enzymes (e.g. trypsin), the determination of peptide masses by mass spectrometry and the matching of the masses against theoretical peptide libraries generated from protein sequence databases to create a list of likely protein identifications. The great advantage of this approach is the low amount of purified protein required.

The specific protein bands from the Coomassie stained SDS-PAGE (Fig. 3.20 B) were excised, destained and treated for tryptic digestion. Resulting peptides were extracted and analyzed by MALDI-TOF MS (Matrix-assisted laser desorption ionization time-of-flight mass spectrometry) to determine the molecular weights of all the components. A MALDI-TOF spectrum was acquired for each sample. The result is the average mass spectra illustrated in Figure 3.21 for proteins '**a**' and '**b**', and in Figure 3.22 for proteins '**c**' and '**d**'. While clear mass spectra were obtained for proteins '**b**', '**c**' and '**d**', the resolution was not sufficient to analyze data of protein '**a**' spectrum.

The MS spectra were used to search the compiled NCBI nonredundant protein database (with the program MS-Fit) and the SWISS-PROT database (with the program PepIdent) to obtain the amino acid sequence for each polypeptide and to identify amino acid composition of the proteins. The MS spectrum produced by the protein '**b**' identified the histone H2A protein from *Lycopersicon esculentum* (Tomato), whereas the MS spectrum produced by the protein '**d**' identified the histone H4 from *Lycopersicon esculentum* (Tomato). Three tryptic peptides could be assigned to each protein and Table 3.3 presents the final result of the search. The MS spectrum from the protein '**c**' did not match any protein in both databases, and therefore no protein identification could be achieved.

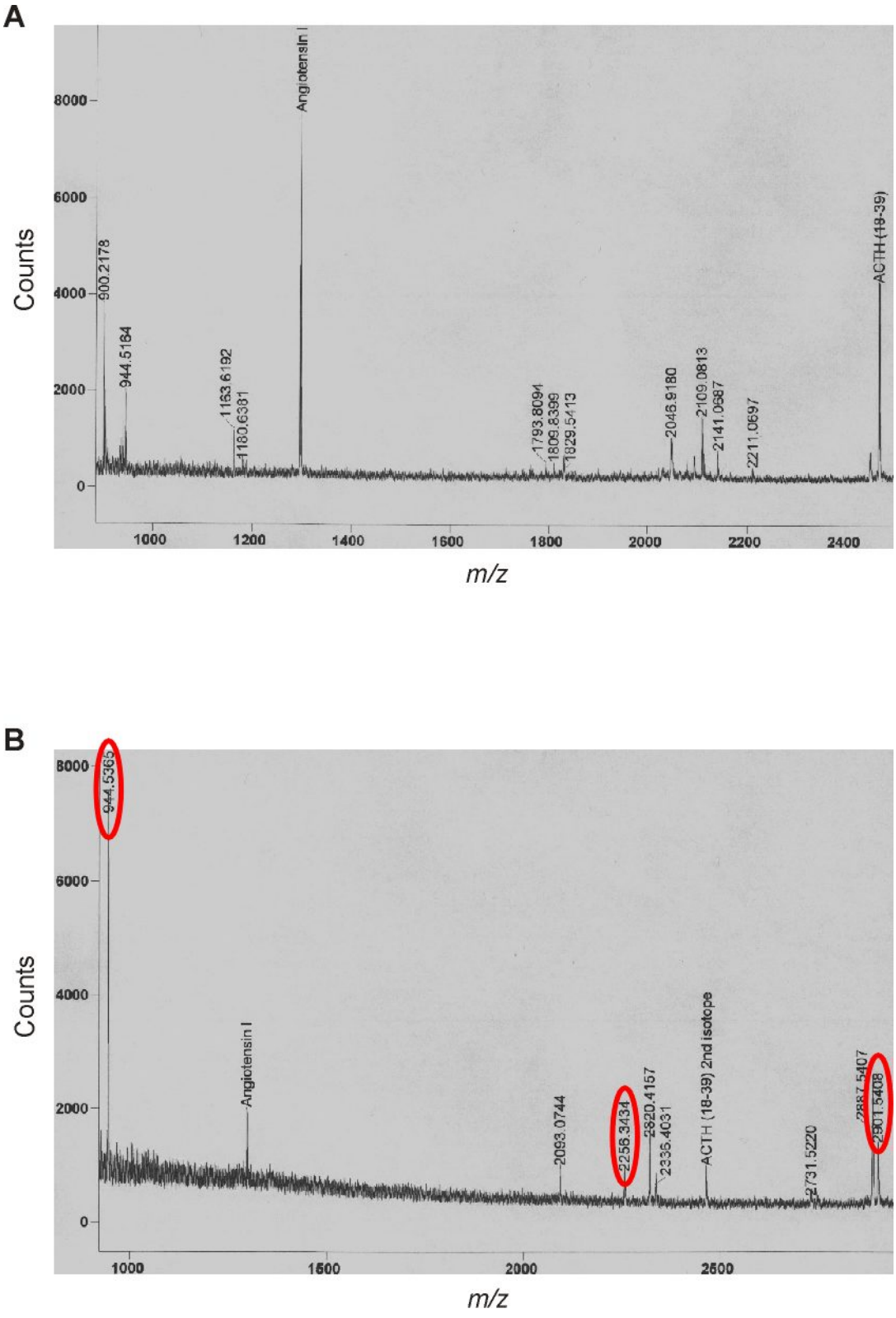
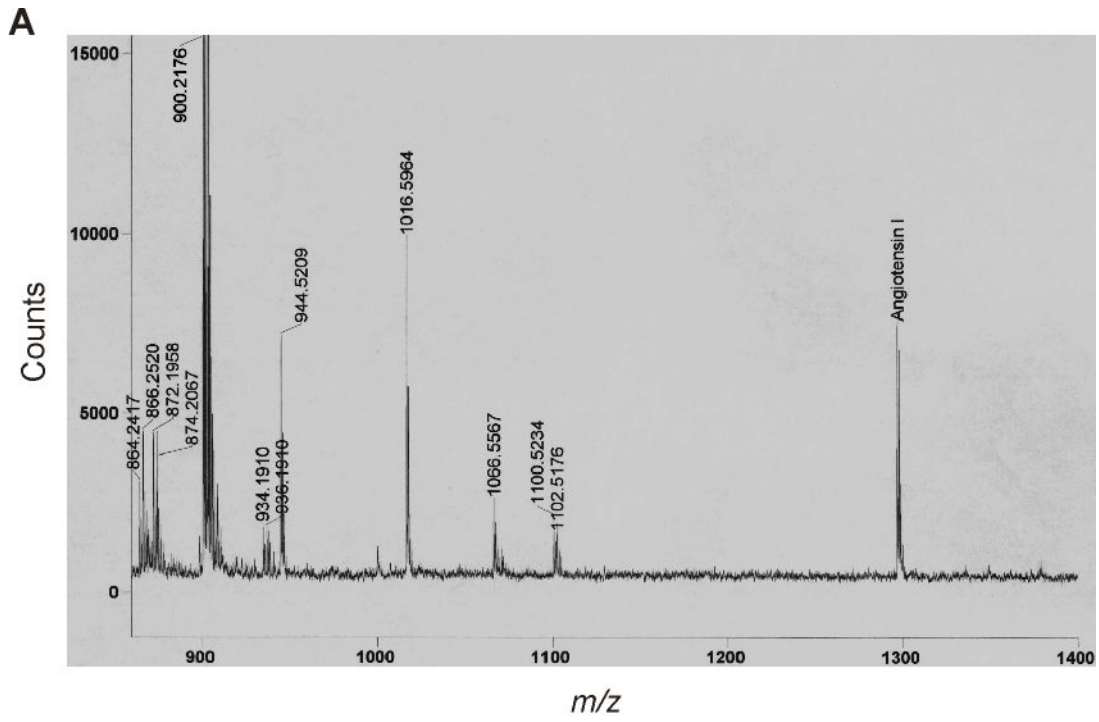


Figure 3.21 MALDI-TOF mass spectrum of peptide fragments produced by trypsin digestion of protein 'a' (A) and protein 'b' (B). Spectra were taken with different intervals. The masses values of the peptides that were later useful for protein identification are in red ellipses. Abbreviation: *m/z*, mass-to-charge ratio. ACTH (acetylcholin) and Angiotensin I are proteins used for calibration.



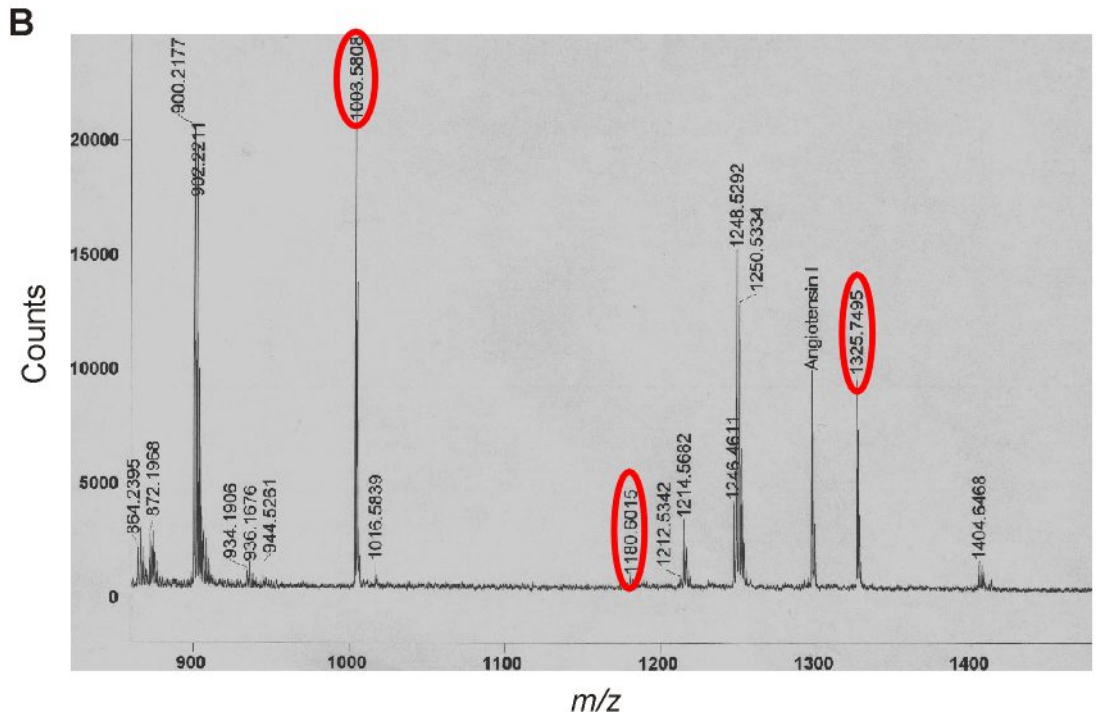


Figure 3.22 MALDI-TOF mass spectrum of peptide fragments produced by trypsin digestion of protein 'c' (A) and protein 'd' (B). See legend to Figure 3.21 for further explanations.

A

HISTONE H2A - *Lycopersicon esculentum* (Tomato) pI: 10.54, MW: 15.335				
User mass	matching mass	Δmass(ppm)	position	peptide
944.537	944.531	-6.15	28-36	**AGLQFPVGR**
2256.343	2256.369	11.48	103-125	**LLAGVTIASGGVLPNINPVLLPK**
2901.451	2901.572	10.67	50-78	**VGSGAPIYLAAVLEYLAAEVLEL AGNAAR**

41.8% of sequence covered:

```
   1          11         21         31         41         51
   |          |          |          |          |          |
 1 mdatkttkga ggrkggprkk svtksikAGL QFPVGRigry lkkgryaqrV GSGAPIYLAA   60
61 VLEYLAAEVL ELAGNAARdn kksriiprhv llavrndeel gkLLAGVTIA SGGVLPNINP  120
121 VLLPKksava eekspkakag kspkka
```

B

HISTONE H4 – *Lycopersicon esculentum* (Tomato) pI: 11.48, MW: 11.294				
User mass	matching mass	Δmass(ppm)	position	peptide
1003.581	1003.593	12.43	60-67	**IFLENVIR**
1180.601	1180.621	16.79	46-55	**ISGLIYEETR**
1325.749	1325.754	3.43	24-35	**DNIQGITKPAIR**

29.4% of sequence covered:

```
   1          11         21         31         41         51
   |          |          |          |          |          |
 1 sgrgkggkgl gkggakrhrk vlrDNIQGIT KPAIRrlarr ggvkrISGLI YEETRgvlkI   60
61 FLENVIRdsv tyteharrkt vtamdvvyal krqgrtlygf gg
```

Table 3.3 Comparison of best matching peptides reveals protein 'b' is histone H2A (A) and protein 'd' is histone H4 (B). The masses values (user mass) of three polypeptide and the matching mass, position and sequence of the derived peptides (residues colored in red), and molecular weight (MW), isoelectric point (pI) and amino acids sequence of the identified proteins are reported. The percentage value indicates the sequence covered. One-letter code for amino acid residues is used.

3.3.2.2 N-terminal ‘sequence tag’ analysis and amino acid composition identification

N-terminal protein sequence analysis was performed for only 5 cycles to create an N-terminal ‘sequence tag’. The chromatographs obtained during the analysis for proteins ‘**b**’ and ‘**d**’ are shown in Figure 3.23 and Figure 3.24: the column elution profile for the last wash (blank), for the amino acids calibration (standard) and for the first five amino acid residues (residue 1 to 5) are reported.

The deduced N-terminal sequence for protein ‘**b**’ is represented by the amino acids:

- A P K A G (Fig. 3.23 B)

Some ambiguity to identify the first residue for protein ‘**d**’ is reported in the chromatograph of Figure 3.24 (Residue 1) since both A and Q might be the possible amino acids; therefore the deduced sequence was:

- A/Q G R G K (Fig. 3.24 B)

Chromatographs of hydrolysate ‘**a**’ and ‘**c**’ bands could not be obtained because of N-terminal blocking.

Protein identification was achieved by comparing the N-terminal ‘sequence tag’ to protein sequences available in databases (NCBI and SWISS-PROT databases). The comparison revealed that the N-terminal ‘sequence tag’ for protein ‘**b**’ matched with the N-terminal sequence of histone H2B (Table 3.4 A) and not with the one of histone H2A as expected according to results obtained by peptide mass finger printing analysis (see Table 3.3 A). This apparently contradictory result may be attributed to the fact that protein ‘**b**’ is a mixture of histone H2A and H2B, which were postranslationally modified (e.g. acetylated or phosphorylated), and therefore co-migrated as proteins of the same size in the SDS-PAGE. Alternatively, technical problems might have been occurred during samples preparation (i.e. cutting out protein bands as gel-slice for MALDI-TOF analysis or extracting proteins from the PVDF membrane for N-

terminal sequencing analysis) since band '**a**' and band '**b**' migrate very close to each other (Fig. 3.20). The N-terminal 'sequence tag' for protein '**d**' matched with the N-terminal sequence of histone H4 (Table 3.4 B) confirming previous results (see Table 3.3 B).

A

N-terminal 'sequence–tag' for protein 'b' : - A P K G A G

HISTONE H2B - *Lycopersicon esculentum* (Tomato) pI: 10.53 , MW: 15.639

```
  1          11         21         31         41         51
  |          |          |          |          |          |
 1 APKAGkkpae kkpveekkae evpaekkpka gkklpkdaga dkkkkkskks vetykiyifk 60
61 vlkqvhpdig issksmgimn sfindifekl aqessrlari nkkptitsre
iqtavrlvlp 120
```

B

N-terminal 'sequence–tag' for protein 'd' : - A/Q G R G K

HISTONE H4- *Lycopersicon esculentum* (Tomato) pI: 11.48, MW: 11.294

```
   1          11         21         31         41         51
   |          |          |          |          |          |
  1 sGRGKggkgl gkggakrhrk vlrdniqgit kpairrlarr ggvkrisgli yeetrgvlki 60
 61 flenvirdsv tyteharrkt vtamdvvyal krqgrtlygf gg
```

Table 3.4 Comparison of N-terminal 'sequence tag' for protein 'b' reveals the best-matching protein is histone H2B (A) whereas the one for protein 'd' reveals the best-matching protein is histone H4 (B). The deduced N-terminal 'sequence tag' for each protein (residues colored in red) and the amino acid sequence for histones H2B and H4 from *Lycopersicon esculentum* (Tomato) are reported. The percentage value indicates the sequence covered. One-letter code for amino acid residues is used

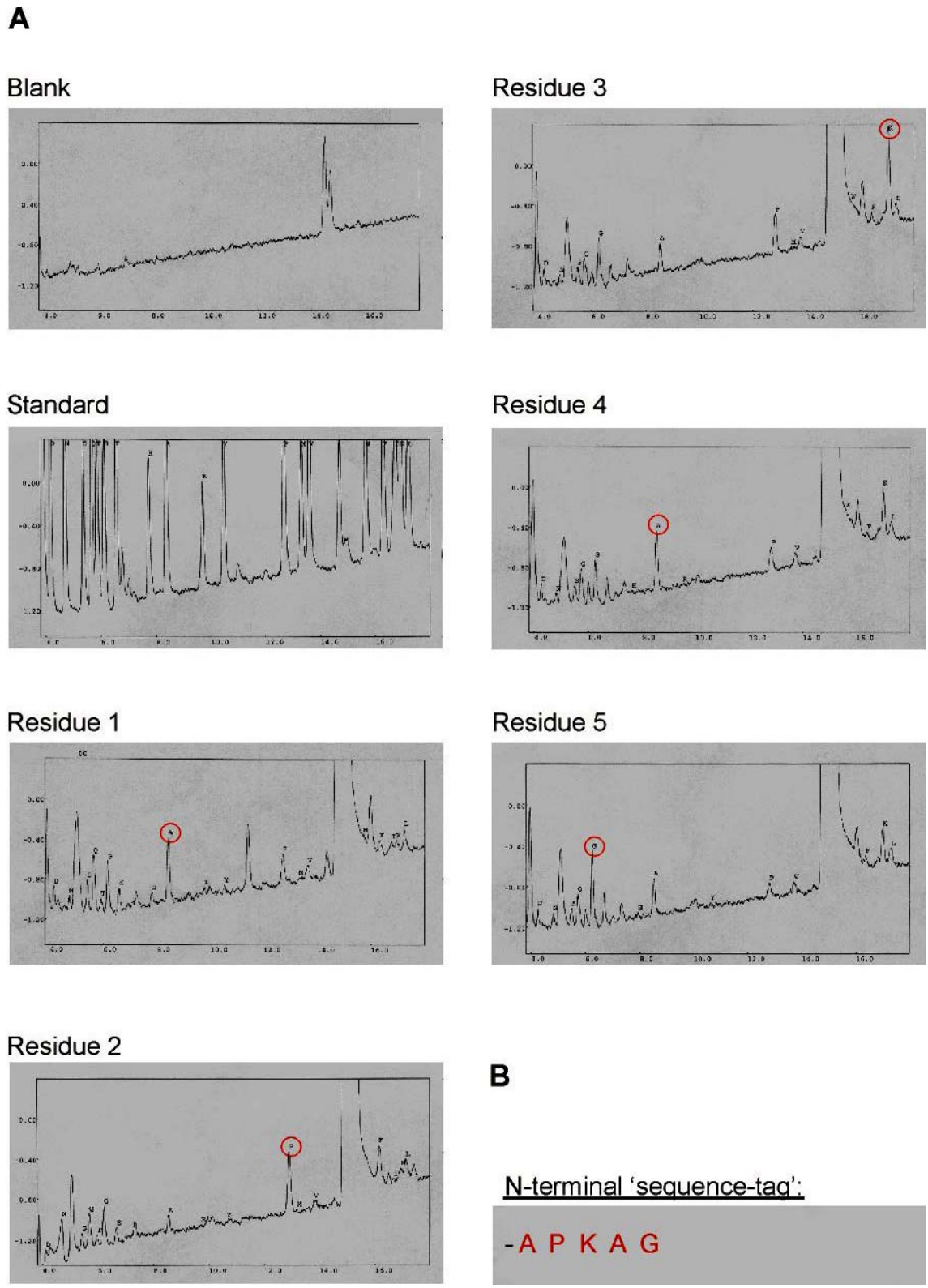


Figure 3.23 Chromatographs of hydrolysate protein 'b' during N-terminal sequencing analysis. The last wash profile (blank), the calibration profile (standard) and the profiles for residue 1 to residue 5 are reported in (**A**), whereas the deduced amino acidic sequence is reported in (**B**). Peaks are labeled with single letter amino acid code; the identified amino acids are in red circles.

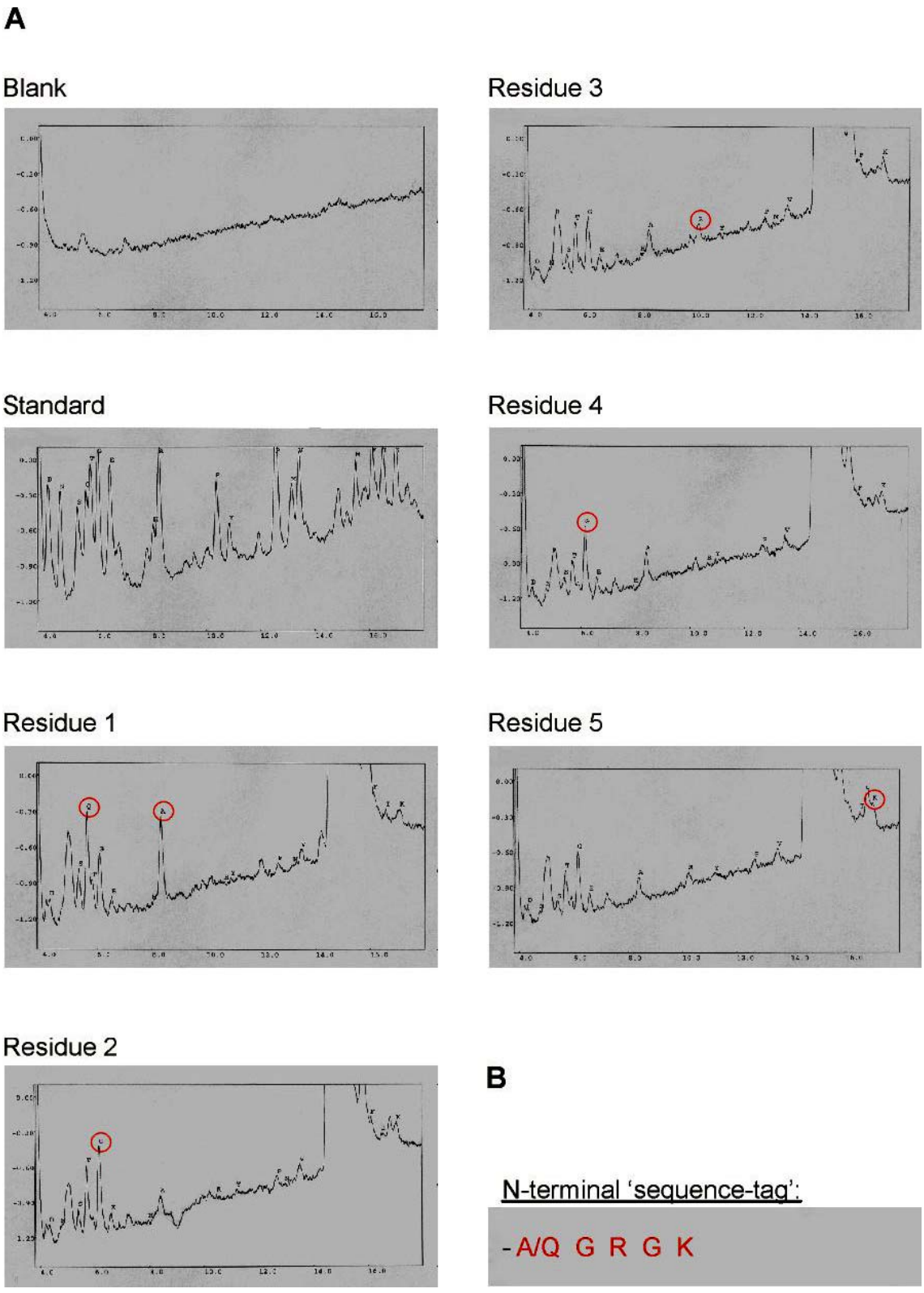


Figure 3.24 Chromatographs of hydrolysate protein 'd' during N-terminal sequencing analysis. See legend to Figure 3.23 for further explanations.

3.3.3 Histones as putative interacting proteins for HsfB1

This study reports the identification of histone H2A, H2B and H4 as proteins specifically associating with tomato HsfB1. This is the first evidence for specific protein interactions of this transcription factor and it is a novel discovery since such interaction was never reported for any other member of the Hsf family.

The histone H2A, H2B and H4, together with histone H3, are the core histone proteins; they play a critical role in the compaction of DNA into nucleosomes and in the overall organization of chromatin (reviewed by Kornberg and Lorch, 1999). Two copies of each histone are assembled into an octamer that has 145-147 base pairs of DNA wrapped around it to form a nucleosome core (Luger et al., 1997). Due to their central role within the cell, these proteins have been very highly conserved throughout evolution. This degree of conservation is exhibited throughout their entire length indicating that there are strict structural constraints on histone function (DeLange et al., 1969, Luger et al., 1997). Therefore, it is not surprising that the short and few peptide sequences obtained by means of MALDI-TOF and N-terminal sequencing analyses permitted to unequivocally identify the histones as HsfB1 interacting proteins (3.3.2). In this work the histone sequences from *Lycopersicon esculentum* have been reported since those from *Lycopersicon peruvianum* were not available in the databases used during the protein identification.

Numerous observations have linked transcriptional activity with the disruption of nucleosomes tight structure within gene promoters. Indeed both *in vitro* and *in vivo* studies indicate that nucleosomes can inhibit transcription initiation by preventing the binding of sequence specific transcription factors to upstream elements and by impeding access of the general transcription machinery to the core promoter (reviewed by Kornberg and Lorch, 1999 and references therein).

The mechanisms by which transcription factors recognize and bind their cognate sites within chromatin are varied. In particular, they can access their sites by aborting the maturation of a nascent nucleosome (preempt mechanism), by outcompeting histones at the replication fork (exclusion mechanism), or by directly invading the preassembled nucleosome (disruption

mechanism) (Workman and Kingston, 1998). In the latter case, transcription factors may be assisted by ATP-dependent chromatin remodeling complexes (Vignali et al., 2000) and/or histone acetyltransferases (Struhl, 1998). Moreover, in recent years, several transcription factors have also been shown to interact directly with histone proteins. Investigations suggest that these proteins may use principles of assembly similar to those observed with the core histones in order to achieve productive protein-protein interactions (Burley et al., 1997; Sullivan et al., 2000).

3.3.4 Histones and HsfB1: what might be the biological relevance of these protein interactions?

Although there are no reports on any physical interaction between a member of the Hsf family and the core histone proteins, several functional clues for such protein interactions might be deduced from other studies which have investigated the mechanisms by which Hsfs bind nucleosomal DNA and influence the chromatin architecture of heat stress promoters.

A notable difference between higher eukaryotes and yeast is that Hsfs have a diverse role in establishing a specific nuclear architecture of the upstream regulatory regions of stress-responsive genes. Particularly in all metazoan heat stress genes which have been examined, the promoter regions are maintained in a transcriptionally poised, nucleosome-free state by other sequence specific regulators, which create an environment conducive for rapid and inducible Hsf binding (Lis and Wu, 1993). In fact Hsf is incapable of binding to the *Drosophila hsp70* promoter in the absence of GAGA, TATA, or initiator elements (Shopland et al., 1995). In addition, *Drosophila* Hsf is incapable of binding even high affinity HSE binding sites assembled into nucleosomes *in vitro* (Becker et al., 1991). Similar results have also been reported for human Hsf (Taylor et al., 1991; Brown et al., 1996). However, other studies revealed an indirect and more general involvement of Hsf in the nuclear architecture. Mizuguchi et al. (1997) showed that in *Drosophila* a nucleosome remodeling factor (NURF) enables Hsf to bind to chromatin templates *in vitro*, whereas other *in vitro* studies, performed using a reconstituted nucleosomal Hsp70 template, have

provided evidence that the human chromatin remodeling complexes SWI-SNF stimulate the ability of the Hsf activation domain to promote elongation (Brown et al., 1996 and 1998). Heat shock promoters of the budding yeast *S. cerevisiae* appear to be maintained in a transcriptionally poised state by Hsf itself (Erkine et al., 1999). This feature underlines the unique role of yeast Hsf in directing basal (nonactivated) transcription and in establishing and maintaining promoters as nucleosome-free regions, e.g. *hsp82* promoter (Gross et al., 1993). Even in the absence of its preferred binding site, Hsf is capable of disrupting the stable nucleosomal structure (Erkine et al., 1996). However, other *in vivo* competition experiments indicate that yeast Hsf binding follows rather than precedes nucleosome formation: nucleosome formation inhibits Hsf binding, although a partial unwrapping of DNA from the edges of already formed nucleosomes allows Hsf binding after nucleosomes assembly (Pederson and Fidrych, 1994; Geraghty et al., 1998). Quite intriguing are the recent findings of Venturi et al. (2000). They have suggested that yeast Hsf might use aspects of the disruption, preempt and exclusion mechanism models with an operative mechanism dependent on the phase of the cell cycle to bind nucleosomal DNA. This proposal is an alternative paradigm for those transcriptional activators which are unable to access their target DNA binding sites when such sites are assembled into stable nucleosomes. Therefore metazoan and yeast Hsfs have a key role in insulating the spread of heterochromatin at the promoter regions of stress-responsive genes and it has never been addressed or reported whether Hsfs can directly interact with the core histone proteins.

Tomato Hsfs have not yet been investigated in respect to their ability to bind or gain access to HSE sites within chromatin. Therefore the identification of the interactions between histones and HsfB1 might indicate that in plant Hsfs can alter nucleosome structure via protein-protein interactions with histones and can directly establish a specific nuclear architecture. This property might be a singular characteristic of HsB1 and some other class B Hsfs.

3.3.5 Several findings hint to peculiarity of HsfB1

Findings presented in this work and several other experimental facts suggest that HsfB1 has unique features compared to class A Hsfs and might modulate critically the transcription of stress-responsive genes during the heat stress response.

- In tomato cell culture HsfB1 is heat stress inducible, it always localizes in the nucleus and its level of expression rapidly decline after heat stress (Scharf et al., 1998b), suggesting a nuclear function during heat stress response.
- HsfB1 but not class A Hsfs interacts with histones (3.3).
- HsfB1 can not interact with class A Hsfs (3.1).
- HsfB1 has a very low transcriptional activator potential, but in tobacco protoplasts a synergistic interaction is seen mainly between tomato HsfA1 and HsfB1 (3.1.5). Moreover, current investigations about the synergistic effect of HsfA1 and HsfB1 in tobacco protoplasts assays suggest that they are particularly efficient on low affinity heat stress promoters and by binding HSE sites in close vicinity indicating a physical interaction between these Hsfs at DNA level (Englich G., Bharti K. and Döring P., unpublished results from our group).
- HsfB1 activity might be regulated by post-translational modifications. Indeed, a putative acetyltransferase motif (Gly-Arg-Gly-Lys) is present in its activation domain. Lys could be the target of acetylation, especially since its mutation to Arg critically reduces the synergistic effect given by coexpressing HsfA1 and HsfB1 (Kumar P., unpublished results from our group). Recently acetylation has been shown to regulate activity of several transcription factors at multiple levels (Soutoglou et al., 2000; Sterner and Berger et al., 2000). Interestingly, HsfA1 but not HsfB1 can recruit components of TFIID and SAGA transcription complexes (Döring P., unpublished results from our group); the latter is also an acetyltransferase-containing complex (Sterner and Berger et al., 2000). It is possible that HsfA1 by recruiting nucleosome-acetylating complexes facilitates the acetylation of HsfB1 and thereby modulates its transcriptional activity.

All these data suggest that during heat stress response HsfB1 might contribute to alter nucleosome structure via protein-protein interactions with histones and establish nucleosome-free regions with new available HSEs sites for Hsfs binding nearby. In addition, given its low activator potential, HsfB1 might be also involved in repression or down-regulation of plant heat stress response, as suggested for other members of plant class B Hsf family (Czarnecka-Verner et al., 2000), for example by preferentially binding to certain HSEs architectures and/or competing with class A Hsfs for DNA-binding.

4 Conclusions

4.1 Some more concepts and future work

4.1.1 Pull-down and yeast two-hybrid assays are suitable methods to study protein interactions of tomato Hsfs

The pull-down and two-hybrid assays have been shown to be powerful methods to identify and characterize protein interactions occurring *in vitro* and *in vivo* respectively (Ratner, 1974; Fields and Song, 1989; Formosa et al., 1991). In particular, throughout this work, the pull-down assay has been chosen because it permits to test all proteins together in an extract. Thus, whole cell extract proteins that are detected have successfully competed for the specific tagged protein (e.g. His_6-Hsf or GST-sHsp) with the rest of the population of proteins. Moreover, interactions that depend on multisubunit tethered proteins (e.g. trimeric Hsf) can be detected. The latter are unlikely to be detected in the two hybrid assay because the interactions take place only between two proteins. However, the pull-down assay does not allow to exclude that other polypeptides, present in the whole cell extract, might mediate or stabilize the protein interactions (e.g. HsfB1 and histones interaction, see 4.1.3). The two-hybrid assay allows to detect interactions that are below the affinity detectable by the pull-down assay (Estojak et al., 1995). However, known protein interactions sometimes can not be analyzed with this approach (Dang et al., 1991; Xia et al., 1994). In this study, an example is HsfB1, whose dimeric interaction was not observed: several mechanisms can be invoked to explain this failure, including conformational incompatibility (e.g. aberrant conformation of hybrid proteins because fused to Gal4 domains or lack of post-translational modifications, which are not provided by the host cell) and inappropriate subcellular localization of the HsfB1 hybrid protein. Western analysis of extracts from yeast cells transformed with plasmids encoding HsfB1 hybrid proteins excluded protein expression and instability as reason for absence of interactions (data not shown).

Given the limitations and advantages of these two methods, both should be used in parallel, whenever possible, to show and confirm protein interactions. This work confirms that both methods are reliable to study most protein-protein interactions of tomato Hsfs and they should be considered simple and valid tools to investigate further protein interactions of the tomato Hsf family.

4.1.2 Remarks about the protein-protein interactions of tomato Hsfs

This study shows that point mutations and deletions in the HR-B region of the oligomerization domain of HsfA2 can critically compromise the interactions between Hsf proteins. Moreover the linker between HR-A and HR-B regions might not have a direct physical role in the protein interactions. These findings should also be assessed in the context of the full length and no hybrid HsfA2 protein to observe if and to what extent these mutations really affect the trimerization state of the protein. Cross-linking and native gel electrophoresis analyses of purified proteins, expressed in bacteria, yeast or in tobacco protoplasts, might provide further evidence for the presented results.

Most of the mutations were performed only in HsfA2 because the high homology of the oligomerization domain of class A Hsfs suggests that they will behave similarly. However, the conclusions about the effects of these mutations on the general oligomerization state of class A Hsfs will be more definitive analyzing also HsfA1 and HsfA3 in this respect.

The hypothesis that the linker region is the domain which ensures stable trimerization and formation of homo- and heterocomplexes of class A Hsfs might be tested in other plant class A Hsfs using assays described in this thesis. Conversely, all class B Hsfs, such as HsfB1, should oligomerize as dimers and not interact with class A Hsfs.

Apparently homologous and heterologous Hsfs complexes are not that relevant to the modulation of the transcriptional activity of a Hsf-dependent reporter gene. Analyses of other Hsf-dependent reporter genes, of the nuclear localization and DNA-binding of the proteins examined (by means of EMSA and DNase-footprinting analyses) should lead to more detailed conclusions.

4.1.3 Confirmation of protein interactions between HsfB1 and histones

The identification of histones as HsfB1 interacting proteins needs further investigations to confirm this result and to establish its biological significance. First, it should be substantiated that histones directly interact with HsfB1. Coimmunoprecipitation assays might provide this evidence and further show that the interactions occur *in vivo*. Second, mutation analyses have to be used to characterize the interactions. Finally functional characterization might start by evaluating DNA-binding ability of HsfB1 on naked DNA and native chromatin (EMSA and DNase-footprinting analyses).

4.1.4 Nature of the protein interaction between HsfA2 and tomato sHsp(II)

Further experiments are needed to characterize the specificity and nature of the interaction between HsfA2 and tomato sHsp(II). Mutation analyses might prove that the N-terminal domain of sHsp(II) is the interacting region with HsfA2 as proposed in the Results and discussions (3.2.3). Coimmunoprecipitation and cross-linking experiments with tomato whole cell extracts from heat stressed cells will indicate whether the interaction occurs *in vivo* and what is the oligomerization state of HsfA2 interacting with sHsp(II). Further work should also investigate whether HsfA2 is a partially denaturated protein during the interaction with sHsp(II) and whether this interaction has a protective role for HsfA2. Alternatively, this interaction may be part of a mechanism regulating the plant heat stress response (negative regulation of HsfA2 by interaction with sHsp(II), see 3.2.4).

4.1.5 The novel class IIs small heat stress protein - a molecular “bridge” between class I and II sHsps

The novel “class IIs” (Lp-Hsp17(IIs)) was originally identified during a yeast two-hybrid screening using full length pea class I sHsp as bait and a heat stress tomato cDNA library (unpublished results from our group). Mainly cDNA

fragments coding for sHsp proteins belonging to the same class as the bait were isolated. Same results were also obtained during another yeast two-hybrid screening with tomato class II sHsp as bait. Lp-Hsp17(IIs) presents higher amino acid sequence identity to class II than class I small heat stress proteins; however, striking is the divergence at the N-terminal domain (Fig. 3.15) indicating that this protein belongs to a novel class and consequently extends the classification of cytoplasmic sHsps (Scharf et al., 2001). Further peculiarity of sHsp(IIs) is shown by the fact that it can interact with both classes of cytoplasmic sHsps (Fig. 3.14). Interestingly, both class I and class II sHsps colocalize in the HSGs (Scharf et al., 1998b; Kirschner et al., 2000), whose formation has been shown to be a specific assembly process primarily dependent on the presence of homooligomers of class II sHsps (Kirschner et al., 2000). Therefore, these results suggest that sHsp(IIs) may function as molecular component that promotes and stabilizes the formation of the HSGs by mediating the interaction between class I and class II sHsp proteins or oligomers. Moreover, sHsp(IIs) itself might be one of the not yet identified components required to give the typical morphology of the HSG complexes (Scharf et al., 1998b).

4.2 A summarizing model of protein interactions for tomato Hsfs

Evidently several protein-protein interactions specifically characterize the members of the tomato Hsf family. These findings in combination with data from other studies offer new insights into the biological role of each tomato Hsfs and support the concept of a Hsfs network during plant heat stress response. A tentative simplified model of cross talk between tomato Hsfs and Hsps via their protein-protein interactions can be summarized as follows (Fig. 4.1):

- Class A Hsfs are trimeric proteins, which interact with each other via the oligomerization (HR-A/B) domain and can form homo- and heterooligomeric complexes (3.1). Trimers, homo- and heterooligomeric complexes might modulate the availability and stoichiometry of active Hsfs-DNA binding

complexes in the nucleus and thereby differentially activate stress-responsive genes during heat stress.

- HsfB1 is a dimer and can not interact with class A Hsfs (3.1). It has a low transcriptional activator potential, but binding of HsfB1 and HsfA1 at adjacent HSE sites on promoters results in a synergistic effect (3.1.5) indicating protein interaction at the DNA level. HsfA1 might also modulate HsfB1 activity indirectly by recruiting other proteins which then interact with HsfB1. Moreover, to date, HsfB1 is the only identified Hsf that interacts with histones (3.3) suggesting that during heat stress HsfB1 can independently bind HSEs sites positioned within nucleosomes, since its DNA-protein interaction is stabilized by the interaction with histones, and maybe establish nucleosome-free regions with new available HSEs sites for Hsfs binding nearby. The final event would be that HsB1 can modulate and increase transcriptional activity of stress-responsive genes.

- HsfA2 is a “shuttling” protein with dominant cytoplasmic localization as a balance of a nuclear import combined with an efficient export (Heerklotz et al., 2001). During heat stress this balance might be directly influenced by several intra- and intermolecular interactions of HsfA2. A temperature dependent conformational change of HsfA2 (i.e. intramolecular interaction between the internal HR-A/B and C-terminal HR-C regions) might shield the NLS motif and impede its nuclear localization as proposed by Heerklotz et al. (2001). However, this structural constrain might also be relieved by its interaction with HsfA1 and HsfA3, which lead to nuclear localization of HsfA2 and specific activation of small heat stress genes. Later, as soon as the cell has reached a Hsps amount that exceeds its cellular demand, sHsp(II) interacts with HsfA2 and might determine or facilitate its localization in the HSGs. Interestingly, HsfA2 can interact both with class A Hsfs and sHsp(II) via the oligomerization domain (3.1 and 3.2), therefore sHsp(II) might compete with HsfA1 to retain HsfA2 in the cytoplasm. The final event might down-regulate the small heat stress genes activation and in general the heat stress response.

- Molecular chaperones, such as Hsp90 and Hsp70, can interact with class A Hsfs (1.5.4) and might negatively regulate their activities as shown for Hsfs in eukaryotes. Moreover, sHsp(II) might specifically and negatively regulate HsfA2 activity.

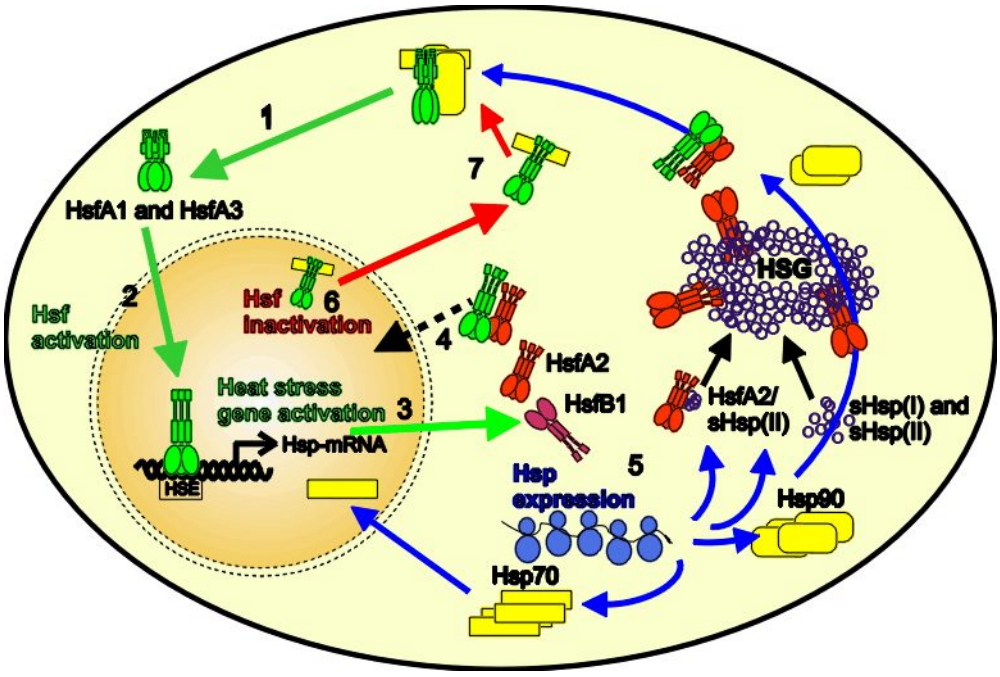


Figure 4.1 Model of cross talk between tomato Hsfs and Hsps via their protein-protein interactions during heat stress response in a tomato cell. During a period of cellular stress, the concentration of non-native proteins increases and molecular chaperones are required to prevent the appearance of unfolded/misfolded proteins. As the cellular level of Hsp70 and Hsp90 chaperones become limiting, the influence that they normally exert on constitutive Hsfs (HsfA1 and HsfA3) is relived (step 1), allowing active HsfA1 and HsfA3 to induce transcription of heat stress genes (steps 2 and 3). HsfA2 and HsfB1 are expressed. Both HsfA1 and HsfA3 mediate a fast HsfA2 nuclear localization (step 4), which specifically mediate transcription of sHsps. It follows a cycle of heat stress gene activation which is fine tuned by the presence of all four tomato Hsfs and by the formation of homo- and heterooligomeric complexes among class A Hsf trimers (steps 1 to 4). The transcriptional activation of the heat stress genes subsequently provides the cell with an Hsps amount that exceeds its cellular demand. The sHsps amount is higher then the need in the cytoplasm, and sHsp(II) competes with the other class A Hsfs to bind HsfA2 and to retain and localize it in the HSG complexes (step 5). This event stops small heat stress genes induction and starts the negative regulation of the plant heat stress response. In parallel Hsp70 and Hsp90 facilitate the conversion of the active forms of Hsfs to inactive forms (chaperones interact again with Hsfs, steps 6 to 1) during recovery, leading to final repression of the heat stress response. (modified from Scharf et al., 1998a)

5 Summary/Zusammenfassung

5.1 Summary

The heat stress response is characterized by the presence of heat stress transcription factors (Hsfs) which mediate transcription of heat stress genes.

In tomato (*Lycopersicon peruvianum*) cell cultures the simultaneous expression of four Hsfs, which are either constitutively (HsfA1 and HsfA3) or heat-stress inducible (HsfA2 and HsfB1) expressed, results in a complex network with dynamically changing cellular levels, intracellular localization and functional interactions. In order to examine the relevance of their multiplicity as well as to get more insights into the complexity of the plant heat stress response, the individual tomato Hsfs were investigated with respect to their protein interactions *in vitro* and *in vivo*. To this aim, I used pull-down assays as well as yeast assays to study the following aspects:

1. Oligomeric state of Hsfs: the results show that all class A Hsfs (HsfA1, HsfA2 and HsfA3) are trimeric proteins and interact with each other via the oligomerization (HR-A/B) domain. The similarity of their HRA/B regions allows formation of homo- and heterooligomeric complexes between all class A Hsfs. This special property was investigated by mutational studies with HsfA2 indicating that the linker and the HR-B regions are the minimal part required for Hsf/Hsf interactions. The conserved hydrophobic amino acid residues of the HR-B region are most important whereas the amino acid residues of the linker may provide higher flexibility to the HR-B region. Another investigated factor was HsfB1. HsfB1 is a member of class B Hsfs, which are characterized by an oligomerization domain without the 21 amino acid residues linker inserted between the HR-A and HR-B regions. It has a low activator potential and exists exclusively as dimer. HsfB1 can not physically interact with class A Hsfs. However, HsfB1 and HsfA1, binding to adjacent HSE sites, are assumed to cause strong synergistic effects in gene activation.

2. Potential HsfB1 interacting proteins: we searched for HsfB1 interacting proteins by using recombinant His-tagged proteins with HsfB1 as baits in pull-down assays. Histones H2A, H2B and H4 were identified by means of Peptide Mass Finger Printing and N-terminal sequencing analyses. The three histones represent the major proteins in tomato whole cell extracts retrieved by HsfB1.

3. HsfA2/small heat stress proteins (sHsps) interaction: pull-down and yeast two-hybrid assays were used to study the specific interaction of HsfA2 with tomato class II sHsp. This interaction occurs via the oligomerization domain of HsfA2. Other members of the plant Hsp20 family, including class I sHsp, do not interact with HsfA2. Heterooligomers of HsfA2 with class II sHsp may represent precursor forms of the plant higher molecular weight cytoplasmic complexes of heat stress granules, which form during heat stress.

The findings presented in this thesis are a contribution to support the concept of a Hsfs network via protein-protein interactions. These data, together with information obtained from other studies, are used to propose a tentative model of the complex Hsfs network controlling the plant heat stress response.

5.2 Zusammenfassung

Hitzestresstranskriptionsfaktoren (Hsfs) sind die zentralen Kontrollfaktoren der Hitzestressantwort in Eukaryoten.

In Tomatenzellkulturen (*Lycopersicon peruvianum*) sind unter Hitzestressbedingungen vier Hsfs detektierbar: die konstitutiv exprimierten Faktoren HsfA1 und HsfA3 und die hitzestressinduzierbaren Faktoren HsfA2 und HsfB1. Die simultane Expression dieser Hsfs führt zu einem regulatorischen Netzwerk, das durch dynamische Veränderungen in der intrazellulären Lokalisation der Hsfs und durch Interaktion untereinander und mit anderen Proteinen gekennzeichnet ist. Um die intramolekularen Wechselwirkungen dieser pflanzlichen Hsfs näher zu untersuchen, wurden in der vorliegenden Arbeit sowohl *in vitro* als auch *in viv*o Interaktionsstudien mit Hilfe von „pull-down" Experimenten, sowie dem Hefe-2-Hybridsystem durchgeführt. Folgende Aspekte wurden näher untersucht:

1. Darstellung des Oligomerisierungszustands der Hsfs: Es konnte gezeigt werden, dass alle Hsfs der Klasse A (HsfA1, HsfA2 und HsfA3) als Trimere vorliegen, die über ihre Oligomerisierungsdomäne (HR-A/B) miteinander in Wechselwirkung treten. Innerhalb der Oligomerisierungsdomäne (HR-A/B Region) befinden sich zahlreiche hydrophobe Aminosäurereste. Hierdurch kommt es zur Bildung von Homo- und Heterokomplexen zwischen Hsfs der Klasse A. Diese einzigartige Fähigkeit, die nicht bei Hsfs anderen Organismen vorkommt, wurde durch Analyse verschiedener HsfA2 Mutanten näher untersucht. Hierbei stellte sich heraus, dass die „Linker-" und die „HR-B-Region" die Minimaldomäne darstellen, um eine entsprechende Wechselwirkung mit anderen Hsfs einzugehen. Die hochkonservierten hydrophoben Aminosäurereste der HR-B-Region sind für das Zustandekommen und die Stabilisierung von Hsf-Komplexen verantwortlich. Die Linker-Region ist nicht direkt an Interaktionen beteiligt aber sie ist offensichtlich wichtig für die Flexibilität der HR-B-Region. Ein weiterer Hsf, der untersucht wurde, ist HsfB1. Dieser Hsf ist ein Mitglied aus der Klasse der B-Hsfs, die sich durch das Fehlen der Linker-Region von 21 Aminosäuren auszeichnen und ein geringes Transaktivierungspotential

aufweisen. Es konnte gezeigt werden, dass HsfB1 ausschliesslich Homodimere ausbildet und nicht mit Hsfs aus der A-Klasse interagiert.

2. Identifizierung von potentiellen Interaktionsproteinen von HsfB1: Zur Suche nach unbekannten HsfB1 Interaktionspartnern, wurden mit rekombinanten HsfB1 als Köder „pull-down“ Experimente durchgeführt. Die Histone H2A, H2B und H4 wurden mit Hilfe von Peptide Mass Finger Printing Analyse und N-terminaler Sequenzanalyse identifiziert. Im Gesamtzellextrakt von Tomatenzellen wurden hauptsächlich diese drei Histone durch HsfB1 gebunden.

3. Interaktion von HsfA2 und niedermolekulare Hitzestressproteine (sHsps). Es konnte gezeigt werden, dass HsfA2 über seine Oligomerisierungsdomäne mit zytoplasmatischen sHsp der Klasse II aus Tomate interagieren kann. Andere pflanzliche sHsps, einschliesslich sHsp der Klasse I können nicht mit HsfA2 interagieren. Die Komplexe aus HsfA2 und sHsp der Klasse II könnten vermutlich eine Vorstufe der pflanzlichen hochmolekularen Hitzestressgranula darstellen, die unter Stressbedingungen ausgebildet wird.

Die Ergebnisse der vorliegenden Arbeit stellen einen wichtigen Schritt zum Verstehen des komplexen Netzwerks der Hsfs und der mit ihnen verbundenen Koregulatoren dar. Zusammen mit anderen Befunden aus Arbeiten in unserer Gruppe wurde ein Modell für das komplexe Netzwerk regulatorischer Proteine in der pflanzlichen Hitzestressantwort entwickelt.

References

Abravaya, K. A., Myers, M., Murphy, S. P., and Morimoto, R. I. (1992). The human heat shock protein Hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes & Dev.* 6: 1153-1164.

Alastalo, T.-P., Lönnström, M., Leppä, S., Kaarniranta, K., Pelto-Huikko, M., Sistonen, L., and Parvinen, M. (1998). Stage-specific expression and cellular localization of the heat shock factor 2 isoforms in the rat seminiferous epithelium. *Exp. Cell Res.* 240: 16-27.

Ali, A., Bharadwaj, S., O'Carroll, R., and Ovsenek, N. (1998). HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Mol. Cell. Biol.* 18: 4949-4960.

Amin, J., Ananthan, J., and Voellmy, R. (1988). Key features of heat shock regulatory elements. *Mol. Cell. Biol.* 8: 3761-3769.

Amin, J., Fernandez, M., Ananthan, J., Lis, J. T., and Voellmy, R. (1994). Cooperative binding of heat shock transcription factor to the *hsp70* promoter *in vivo* and *in vitro*. *J. Biol. Chem.* 269: 4804-4811.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1993). Current protocols in molecular biology. (eds) J. Wiley & Sons.

Baler, R., Welch, W. J., and Voellmy, R. (1992). Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *J. Cell. Biol.* 117: 1151-1159.

Baler, R., Dahl, G., and R. Voellmy. (1993). Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor. *Mol. Cell. Biol.* 13: 2486-2496.

Baler, R., Zou, J., and Voellmy, R. (1996). Evidence for a role of Hsp70 in the regulation of the heat shock response in mammalian cells. *Cell Stress & Chap.* 1: 33-39.

Ballester, R., Michaeli, T., Ferguson, K., Xu, H. P., McCormick, F., and Wigler, M. (1989). Genetic analysis of mammalian GAP expressed in yeast. *Cell* 59: 681-686.

Baxevanis, A. D., and Vinson, C. R. (1993). Interactions of coiled coils in transcription factors: where is the specificity ? *Curr. Opin. Gen. Dev.* 3: 278-285.

Becker, P. B., Rabindran, S., and Wu., C. (1991). Heat shock-regulated transcription *in vitro* from a chromatin template. *Proc. Natl. Acad. Sci. USA* 88: 4109-4113.

Becker, P., and Wu, C. (1992). Cell-free system for assembly of transcriptionally repressed chromatin from *Drosophila* embryos. *Mol. Cell. Biol.* 12: 2241-2249.

Berengian, A. R., Parfenova, M., and Mchaourab, H. S. (1999). Site-directed spin labeling study of subunit interactions in the alpha-crystallin domain of small heat-shock proteins. Comparison of the oligomer symmetry in alphaA-crystallin, HSP 27, and HSP 16.3. *J. Biol. Chem.* 274: 6305-6314.

Bharadwaj, S., Ali, A., and Ovsenek, N. (1999). Multiple components of the HSP90 chaperone complex function in regulation of Heat Shock Factor 1 *in vivo*. *Mol. Cell. Biol.* 19: 8033-8041.

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

Bohen, S. P., Kralli, A., and Yamamoto, K. R. (1996). Hold 'em and fold 'em: Chaperones and signal transduction. *Science* 268: 1303-1304.

Bonner, J. J., Ballou, C., and Fackenthal, D. L. (1994). Interactions between DNA-bound trimers of the yeast heat shock factor. *Mol. Cell. Biol.* 14: 501-508.

Boscheinen, O. (1997). Funktionsanalyse von pflanzlichen Hitzestreß transkriptions-faktoren durch Expression in Hefe und *in vitro*-Studien zur Proteininteraktion. PhD thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

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

Brown, S. A., Imbalzano, A. N., and Kingston, R. E. (1996). Activator-dependent regulation of transcriptional pausing on nucleosomal templates. *Genes & Dev.* 10: 1479-1490.

Brown, S. A., and Kingston, R. E. (1997). Disruption of downstream chromatin directed by a transcriptional activator. *Genes & Dev.* 11: 3116-3121.

Brown, S. A., Weirich, C. S., Newton, E. M., and Kingston, R. E. (1998). Transcriptional activation domains stimulate initiation and elongation at different times and via different residues. *EMBO J.* 17: 3146-3154.

Bukau, B., and Horwich, A. L. (1998). The Hsp70 and Hsp60 Chaperone Machines. *Cell* 92: 351-366.

Burkhard, P., Stetefeld, J., and Strelkov, S. V. (2001). Coiled coils: a highly versatile protein folding motif. *Trends in Cell Biology* 11: 82-88.

Burley, S. K., Kie, X., Clark, K. L., and Shu, F. (1997). Histone-like transcription factors in eukaryotes. *Curr. Opin. Struct. Biol.* 7: 94-102.

Chen, A. (1999). Hefe 2-Hybridscreening mit dem HsfA2. Diploma thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

Chen, Q., Osteryoung, K., and Vierling, E. (1994). A 21 kDa chloroplast heat shock protein assembles into high molecular weight complexes *in vivo* and in organelle. *J. Biol. Chem.* 269: 13216-13223.

Chen, Y., Barlev, N. A., Westergaard, O., and Jakobsen, B. K. (1993). Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. *EMBO J.* 12: 5007-5018.

Clos, J., Westwood, J. T., Becker, P. B., Wilson, S., Lambert, K., and Wu, C. (1990). Molecular cloning and expression of hexameric *Drosophila* heat shock factor subjected to negative regulation. *Cell* 63: 1085-1097.

Clos, J., Rabindran, S., Wisniewski, J., and Wu, C. (1993). Induction temperature of human heat shock factor is reprogrammed in a *Drosophila* cell environment. *Nature* 364: 252-255.

Cohen, R. S., and Meselson, M. (1988). Periodic interactions of heat shock transcriptional elements. *Nature.* 332: 856-858.

Cohen, C., and Parry, D. A. D. (1994). α-helical coiled coils: more facts and better predictions. *Science* 263: 488-489.

Cotto, J. J., Kline, M., and Morimoto, R. I. (1996). Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. Evidence for a multistep pathway of regulation. *J. Biol. Chem.* 271: 3355-3358.

Czarnecka-Verner, E., Yuan, C. X., Foxand, P. C., and Gurley, W. B. (1995). Isolation and characterization of six heat shock transcription factor cDNA clones from soybean. *Plant Mol. Biol.* 29: 37-51.

Czarnecka-Verner, E., Yuan, C. X., Scharf, K.-D., Englich, G., and 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.

Damberger, F. F., Pelton, J. G., Harrison, C. J., Nelson, H. C. M., and Wemmer, D. E. (1994). Solution structure of the DNA-binding domain of the heat shock transcription factor determined by multidimensional heteronuclear magnetic resonance spectroscopy. *Protein Sci.* 3: 1806-1821.

Dang, C. V., Barrett, J., Villa-Garcia, M., Reasar, L. M. S., Kato, G. J., and Fearon, E. R. (1991). Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell. Biol.* 11: 954-962.

DeLange, R. J., Fambrough, D. M., Smith, E. L., and Booner, J. (1969). Calf and pea histone IV. 3. Complete amino acid sequence of pea seedling histone IV; comparison with the homologous calf thymus histone. *J. Biol. Chem.* 244: 5669-5679.

DeJong, W. W., Leunissen, J. A., and Vooter, C. E. (1993). Evolution of the α-crystallin /small heat-shock protein family. *Mol. Biol. Evol.* 10: 103-126.

Döring, P., Treuter, E., Kistner, C., Lyck, R., Chen, A., and Nover, L. (2000). The role of AHA motifs in the activator function of tomato heat stress transcription factors HsfA1 and HsfA2. *Plant Cell.* 12: 265-278.

Drees, B. L., Grotkopp, E. K., and Nelson, H. C. M. (1997). The GCN4 leucine zipper can functionally substitute for the heat shock transcription factor's trimerization domain. *J. Mol. Biol.*: 273: 61-74.

Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997). Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *EMBO J.* 16: 221-229.

Erkine, A. M., Adams, C. C., Diken, T., and Gross, D. S. (1996). Heat shock factor gains access to the yeast *hsc82* promoter independently of other sequence-specific factors and antagonizes nucleosomal repression of basal and induced transcription. *Mol. Cell. Biol.*16: 7004-7017.

Erkine, A. M., Magrogan, S. F., Sekinger, E. A., and Gross, D. S. (1999). Cooperative binding of heat shock factor to the yeast *hsp*82 promoter *in vivo* and *in vitro. Mol. Cell. Biol.* 19: 1627-1639.

Estojak, J., Brent, R., and Golemis, E. A. (1995). Correlation of two-hybrid affinity data with *in vitro* measurements. *Mol. Cell. Biol.* 15: 5820-5829.

Farkas, T., Kutskova, Y. A., and Zimarino, V. (1998). Intramoleculaar repression of mouse heat shock factor 1. *Mol. Cell. Biol.* 18: 906-918.

Fernandes, M., Xiao, H., and Lis, J. T. (1995). Binding of heat shock factor to and transcriptional activation of heat shock genes in *Drosophila*. *Nucleic Acids Res.* 23: 4799-4804.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* 340: 245-247.

Fiorenza, M. T., Farkas, T., Dissing, M., Kolding, D., and Zimarino, V. (1995). Complex expression of murine heat shock transcription factors. *Nucleic Acids Res.* 23: 467-474.

Forreiter, C., Kirschner, M., and Nover, L. (1997). Stable transformation of an *Arabidopsis* cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity *in vivo*. *Plant Cell.* 9: 2171-2181.

Forreiter, C., and Nover, L. (1998). Heat induced stress proteins and the concept of molecular chaperones. *J. Biosci.* 23: 287-302.

Formosa, T., Barry, J., Alberts, B. M., and Greenblatt, J. (1991). Using protein affinity chromatography to probe structure of protein machines. *Methods Enzymol.* 208: 24-45.

Frangioni, J. V., and Neal, B. G. (1993). Solubilization and purification of enzymatically active Glutahione S-Transferase (pGEX) fusion proteins. *Analytical Biochemistry* 210: 179-187.

Gallo, L., Prentice, H., and Kingston, R. E. (1993). Heat shock factor is required for growth at normal temperatures in fission yeast *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 13: 749-761.

Geraghty, D. S., Sucic, H. B., Chen, J., and Pederson, D. S. (1998). Evidence that partial unwrapping of DNA from nucleosomes facilitates the binding of heat shock factor following DNA replication in yeast. *J. Biol. Chem.* 273: 20463-20472.

Goodson, M. L., Park-Sarge, O. K., and Sarge, K. D. (1995). Tissue-dependent expression of heat shock factor 2 isoforms with distinct transcriptional activities. *Mol. Cell. Biol.* 15: 5288-5293.

Gross, D. S., Adams, C. C., Lee, S., and Stentz, B. (1993). A critical role for heat shock transcription factor in establishing a nucleosome-free region over the TATA-initiation site of the yeast *hsp82* heat shock gene. *EMBO J.* 12: 3931-3945.

Haley, D. A., Bova, M. P., Huang, Q.-L., Mchaourab, H. S., and Stewart, P. L. (2000). Small Heat-Shock Protein structures reveal a continuum from symmetric to variable assemblies. *J. Mol. Biol.* 298: 261-272.

Hanahan, D. (1983). Studies on trasformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-560.

Harbury, P. B., Zhang, T., Kim, P. S., and Alber, T. (1993). A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* 262: 1401-1407.

Harbury, P. B., Kim, P. S., and Alber, T. (1994). Crystal structure of an isoleucine-zipper trimer. *Nature* 371: 80-83.

Harrison, C. J., Bohm, A. A., and Nelson, H. C. M. (1994). Crystal structure of the DNA binding domain of the heat shock transcription factor. *Science* 263: 224-227.

Hartl, F. U. (1996). Molecular chaperones in cellular folding. *Nature* 381: 571-579.

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

Helm, K. W., Lee, G. J., and Vierling, E. (1997). Expression and native structure of cytosolic class II small heat shock proteins. *Plant Physiol.* 114: 1477-1485.

Hendsch, Z. S., Jonsson, T., Sauer, R. T., and Tidor, B. (1996). Protein stabilization by removal of unsatisfied polar groups: computational approaches and experimental tests. *Biochemistry* 35: 7621-7625.

Hoj, A., and Jakobsen, B. K. (1994). A short element required for tuning off heat shock transcription factor: evidence that phosphorylation enhances deactivation. *EMBO J.* 13: 2617-2624.

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

Hübel, A., and Schöffl, F. (1994). *Arabidopsis* heat shock factor: isolation and characterization of the gene and the recombinant protein. *Plant Mol. Biol.* 26: 353-362.

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (1990). PCR protocols: a guide to methods and applications. Academic Press Inc., New York J. Biol. Chem. 274: 6305-6314.

Jakobsen, B. K., and Pelham, H. R. (1991). A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. *EMBO J.* 10: 369-375.

Janista, C. (1999). Mutagenese und Funktionsanalyse der Oligomerisierungsdomäne des Hitzestreßtranskriptionsfaktors HsfA2. Diploma thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

Jedlicka, P., Mortin, M. A., and Wu, C. (1997). Multiple functions of *Drosophila* heat shock transcription factor *in vivo*. *EMBO J.* 16: 2452-2462.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: The *gus* gene fusion system. *Plant Mol. Biol. Rep.* 5: 387-405.

Jinn, T., Chem, Y., and Lin. C. (1995). Characterization and physiological function of class I low molecular mass, heat shock protein complex in soybean. *Plant Physiol.* 108: 693-701.

Kanei-Ishii, C., Tanikawa, J., Nakai. A., Morimoto, R., and Ishii, S. (1997). Activation of the heat shock transcription factor 3 by c-Myb in the absence of cellular stress. *Science* 277: 246- 248.

Kim, K. K., Kim, R., and Kim, S. H. (1998). Crystal structure of a small heat-shock protein. *Nature* 394: 595-599.

Kirschner, M., Winkelhaus, S., Thierfelder, J. M., and Nover, L. (2000). Transient expression and heat-stress-induced co-aggregation of endogenous and heterologous small heat-stress proteins in tobacco protoplasts. *Plant J.* 24: 397-412.

Kline, M. P., and Morimoto, R. I. (1997). Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Mol. Cell. Biol.* 17: 2107-2115.

Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., and Aoyama, A. (1991). αB-Crystallin is a small heat-shock protein. *Proc. Natl. Acad. Sci. USA* 88: 3652-3656.

Knauf, U., Newton, E. M., Kyriakis, J., and Kingston, R. E. (1996). Repression of human heat shock factor 1 activity at control temperature by phosphorylation. *Genes & Dev.* 10: 2782-2793.

Kornberg, R. D., and Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* 98: 285-294.

Kraft, M. (1999). Funktionsanalyse von Hsp90 in Pflanzen. Diploma thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

Kroeger, P. E., and Morimoto, R.I. (1994). Selection of new HSF1 and HSF2 DNA-binding sites reveals difference in trimer cooperativity. *Mol. Cell. Biol.* 14: 7592–7603.

Kunert, O. (1997). Untersuchungen zur Struktur und zur DNA-Bindung eines pflanzlichen Hitzestresstranskriptionsfaktors. PhD thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240: 1759-1764.

Lee, G. J., Pokala, N., and Vierling, E. (1995). Structure and *in vitro* molecular chaperone activity of cytosolic small heat shock protein from pea. *J. Biol. Chem.* 270: 10432-10438.

Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J.* 16: 659-671.

Lee, G. J., and Vierling, E. (2000). A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol.* 122: 189-198.

Levchenko, I., Smith, C. K., Walsh, N. P., Sauer, R. T., and Baker, T. A. (1997). PDZ-like domains mediate binding specificity in the Clp/Hsp100 family of chaperones and protease regulatory subunits. *Cell* 91: 939–947.

Lis, J. T., and Wu, C. (1993). Protein traffic on the heat shock promoter: parking, stalling, and trucking along. *Cell* 74: 1-20.

Littlefield, O., and Nelson, H. C. M. (1999). A new use for the 'wing' of the 'winged' helix-turn-helix motif in the HSF–DNA cocrystal. *Nat. Struct. Biol.* 6: 464-470.

Liu, X. D., Liu, P. C. C., Santoro, N., and Thiele, D. J. (1997). Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF. *EMBO J.* 16: 6466-6477.

Liu, P. C. C., and Thiele, D. J. (1999). Modulation of human heat shock factor trimerization by the linker domain. *J. Biol. Chem.* 274: 17219-17225.

Lovejoy, B., Choe, S., Cascio, D., McRorie, D. K., DeGrado, W. F., and Eisenberg, D. (1993). Crystal structure of a synthetic triple-stranded α-helical bundle. *Science* 259: 1288-1293.

Löw, D., Brändle, K., Nover, L., and Forreiter, C. (2000). Cytosolic heat-stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones *in vivo*. *Planta* 211: 575-582.

Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 389: 251-260.

Lumb, K. J., and Kim, P. S. (1995). A buried polar interaction imparts structural uniqueness in a designed heterodimeric coiled coil. *Biochemistry* 34: 8642-8648.

Lupas, A. (1996). Coiled coils: new structures and new functions. *Trends Biochem. Sci.* 21: 375-383.

Lyck, R., Harmening, U., Höhfeld, I., Treuter, E., Scharf, K.-D., and Nover, L. (1997). Intracellular distribution and identification of the nuclear localization signals of two plant heat-stress transcription factors. *Planta* 202: 117-125.

Mason, P. B. J., and Lis., J. T. (1997). Cooperative and competitive protein interactions at the *hsp70* promoter. *J. Biol. Chem.* 272: 33227-33233.

Mathew, A., Mathur, S. K., and Morimoto, R. I. (1998). The heat shock response and protein degradation: regulation of HsfA2 by the ubiquitin-proteosome pathway. *Mol. Cell. Biol.* 18: 5091-5098.

Mezger, V., Rallu, M., Morimoto, R. I., Morange, M., and Renard J. P. (1994). Heat shock factor 2-like activity in mouse blastocysts. *Dev. Biol.* 166: 819-822.

Mishra, S.-K. (2000). Analysis of transgenic Tomato Lines with Changed Expression of Heat Stress transcription Factors. Diploma thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

Mizuguchi, G., Tsukiyama, T., Wisniewski, J., and Wu, C. (1997). Role of nucleosome remodeling factor NURF in transcriptional activation of chromatin. *Mol. Cell.* 1: 141-150.

Morimoto, R. I., Tissierer, A., and Georgopoulos, C. (eds.) (1994). The biology of heat shock proteins and molecular chaperones. New York: Cold Spring Harbor Laboratory Press.

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

Mosser, D. D., Duchaine, J., and Massie, B. (1993). The DNA-binding activity of the human heat shock transcription factor is regulated *in vivo* by hsp70. *Mol. Cell. Biol.* 13: 5427-5438.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

Nadeau, K., Das, A., and Walsh, C. T. (1993). Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J. Biol. Chem.* 268: 1479-1487.

Nair, S. C., Toran, E. J., Rimerman, R. A., Hjermstad, S., Smithgall, T. E., and Smith, D. F. (1996). A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor HSF1, and the aryl hydrocarbon receptor. *Cell Stress & Chap.* 1: 237-250.

Nakai, A., and Morimoto, R. I. (1993). Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol. Cell. Biol.* 13: 1983-1997.

Nakai, A., Kawazoe, Y., Tanabe, M., Nagata, K., and Morimoto, R. I. (1995). The DNA-binding properties of two heat shock factors, Hsf1 and Hsf3, are induced in the avian erythroblast cell line HD6. *Mol. Cell. Biol.* 15: 5268-5278.

Nakai, A., Tanabe, M., Kawazoe, Y., Inazawa, J., Morimoto, R. I., and Nagata, K. (1997). HSF4, a new member of the human heat shock factor family which lacks properties of a transcriptional activator. *Mol. Cell. Biol.* 17: 469-481.

Neumann, D., Scharf, K.-D., and Nover, L. (1984). Heat shock induced changes of plant cell ultrastructure and autoradiographic localization of heat shock proteins. *Eur. J. Cell. Biol.* 34: 254-264.

Nieto-Sotelo, J., Wiederrecht, G., Okuda, A., and Parker, C. S. (1990). The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshocked conditions. *Cell* 62: 807-817.

Nover, L., Kranz, E., and Scharf, K.-D. (1982). Growth cycle of suspension cultures of *Lycopersicon esculentum* and *L. peruvianum*. *Biochem. Physiol. Pflanzen* 177: 483-499.

Nover, L., Scharf, K.-D., and Neumann, D. (1983). Formation of cytoplasmic heat shock granules in tomato cell cultures and leaves. *Mol. Cell. Biol.* 3: 1648-1655.

Nover, L., Neumann, D., and Scharf, K.-D. (1989a). Heat shock and other stress response systems of plants. *Results Probl. Cell. Differ.*16: Berlin, Springer.

Nover, L., Scharf, K.-D., and Neumann, D. (1989b). Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs. *Mol. Cell. Biol.* 9: 1289-1308.

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

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., and Scharf, K.-D. (2001). *Arabidopsis* and the Hsf world: How many heat stress transcription factors do we need? *Cell Stress & Chap., in press.*

Nunes, S. L., and Calderwood, S. K. (1995). Heat shock factor 1 and the heat shock cognate 70 protein associate in high molecular weight complexes in the cytoplasm of NIH-3T3 cells. *Biochem. Biophys. Res. Commun.* 213: 1-6.

Parsell, D. A., and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27: 437-496.

Pederson, D. S., and Fidrych, T. (1994). Heat shock factor can activate transcription while bound to nucleosomal DNA in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 14:189-199.

Perisic, O., Xiao, H., and Lis, J. T. (1989). Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. *Cell* 59: 797-806.

Peteranderl, R., and Nelson, H. C. M. (1992). Trimerization of the heat shock transcription factor by a triple-stranded alpha-helical coiled-coil. *Biochemistry* 31: 12272-12276.

Peteranderl, R., Rabenstein, M., Shin, Y.-K., Corey, W. L., Wemmer, E. D., King, D. S., and Nelson, H. C. M. (1999). Biochemical and biophysical characterization of the trimerization domain from the heat shock transcription factor. *Biochemistry* 38: 3559-3569.

Pirkkala, L., Alastalo, T.-P., Nykänen, P., Seppä, L., and Sistonen, L. (1999). Differentiation lineage-specific expression of human heat shock transcription factor 2. *FASEB J.* 13: 1089-1098.

Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 90: 65–75.

Rabindran, S. K., Giorgi, G., Clos, J., and Wu, C. (1991). Molecular cloning and expression of a human heat shock factor. *Proc. Natl. Acad. Sci.* USA 88: 6906-6910.

Rabindran, S. K., Haroun, R. I., Clos, J., Wisniewski, J., and Wu, C. (1993). Regulation of heat shock factor trimerization: role of a conserved leucine zipper. *Science* 259: 230-234.

Rabindran, S. K., Wisniewski, J., Li, L., Li, G. C., and Wu, C. (1994). Interaction between heat shock factor and Hsp70 is insufficient to suppress induction of DNA-binding activity *in vivo*. *Mol. Cell. Biol.* 14: 6552-6560.

Ratner, D. (1974). The interaction of bacterial and phage proteins with immobilized *Escherichia coli* RNA polymerase. *J. Mol. Biol.* 88: 373-383.

Reindl, A., Schöffl, F., Schell, J., Koncz, C., and Bakò, L. (1997). Phosphorylation by a cyclin-dependent kinase modulates DNA binding of the *Arabidopsis* heat shock transcription Factor Hsf1 *in vitro*. *Plant Physiol.* 115: 93-100.

Reindl, A., and Schöffl, F. (1998). Interaction between the *Arabidopsis thaliana* heat shock transcription factor HSF1 and the TATA binding protein TBP. *FEBS letters* 436: 318-322.

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

Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* 64: 615-623.

Rojas, A., Almoguera, C., and Jordano, J. (1999). Transcriptional activation of a heat shock gene promoter in sunflower embryos: synergism between ABI3 and heat shock factors. *Plant J.* 20: 601-610.

Rose, M. D., Winston, F., Hieter, P. (1990). Methods in yeast genetics. Cold Spring Harbour: Cold Spring Harbor Laboratory Press, NY.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sarge, K. D., Zimarino, V., Holm, K., Wu, C., and 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.

Sarge, K. D., Murphy, S. P., and 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., Park-Sarge, O. K., Kirby, J. D., Mayo, K. E., and Morimoto, R. I. (1994). Expression of heat shock factor 2 in mouse testis: potential role as a regulator of heat-shock protein gene expression during spermatogenesis. *Biol. Reprod.* 50: 1334-1343.

Satyal, S. H., Chen, D., Fox, S. G., Kramer, J. M., and 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., and 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., Rose, S., Thierfelder, J., and Nover, L. (1993). Two cDNAs for tomato heat stress transcription factors. *Plant Physiol.* 102: 1355-1356.

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

Scharf, K.-D., Heider, H., Höhfeld, I., Lyck, R., Schmidt, E., and Nover, L. (1998b). 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., Siddique, M., and Vierling, E. (2001). The expanding family of *Arabidopsis thaliana* small heat stress proteins (sHsps) and a new family of proteins containing α-crystallin domains (Acd proteins). *Cell Stress & Chap., in press.*

Schirmer, E. C., Glover, J. R., Singer, M. A., and Lindquist, S. (1996). Hsp100/Clp proteins: a common mechanism explains diverse functions. *Trends Biochem. Sci.* 21: 289-296.

Schlesinger, M. J., Aliperti, G., and Kelley, P. M. (1982). The response of cells to heat shock. *Trends Biochem. Sci.* 7: 222-225.

Schmidt, E. (1997). Protein-Protein Interaktionsstudien mit dem Two-Hybrid System. Diploma thesis at the J. W. Goethe-Universität Frankfurt am Main, Germany.

Schöffl, F., Rieping, M., Baumann, G., Bevan, M. W., and Angermüller, S. (1989). The function of heat shock promoter elements in the regulated expression of chimaeric genes in transgenic tobacco. *Mol. Gen. Genet.* 217: 246-253.

Schuetz, T. J., Gallo, G. J., Sheldon, L., Tempst, P., and 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.

Schultheiss, J., Kunert, O., Gase, U., Scharf, K.-D., Nover, L., and Rüterjans H. (1996). Solution structure of the DNA-binding domain of the tomato heat stress transcription factor HSF24. *Eur. J. Biochem.* 236: 911-921.

Sheldon, L. A., and Kingston, R. E. (1993). Hydrophobic coiled-coil domains regulate the subcellular localization of human heat shock factor-2. *Genes & Dev.* 7: 1549-1558.

Shi, Y., Kroeger, P. E., and Morimoto, R. I. (1995). The carboxyl-terminal transactivation domain of heat shock factor 1 is negatively regulated and stress responsive. *Mol. Cell. Biol.* 15: 4309-4318.

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

Shopland, L. S., Hirayoshi, K., Fernandes, M., and Lis, J. T. (1995). HSF access to heat shock elements *in vivo* depends critically on promoter architecture defined by GAGA factor, TFIID, and RNA polymerase II binding sites. *Genes & Dev.* 9: 2756-2769.

Shu, W., Ji, H., and Lu, M. (1999). Trimerization specificity in HIV-1 gp41: analysis with a GCN4 leucine zipper model. *Biochemistry* 38: 5378-5385.

Sistonen, L., Sarge, K. D., Phillips, B., Abravaya, K., and Morimoto, R. I. (1992). Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. *Mol. Cell. Biol.* 12: 4104-4111.

Sistonen, L., Sarge, K. D., and Morimoto, R. I. (1994). Human heat shock factors 1 and 2 are differentially activated and can synergistically induce *hsp70* gene transcription. *Mol. Cell. Biol.* 14: 2087-2099.

Smith, B. J., and Yaffe, M. P. (1991). A mutation in the yeast heat-shock factor gene causes temperature-sensitive defects in both mitochondrial protein import and the cell cycle. *Mol. Cell. Biol.* 11: 2647-2655.

Smỳkal, P., Hrdỳ, I., and Pechan, P. M. (2000). High-molecular-mass complexes formed *in vivo* contain smHSPs and HSP70 and display chaperone-like activity. *J. Biochem.* 267: 2195-2207.

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

Sorger, P. K., and Nelson, H. C. M. (1989). Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell* 59: 807-813.

Soutoglou, E., Katrakili, N., and Talianidis, I. (2000). Acetylation regulates transcription factor activity at multiple levels. *Mol. Cell* 5: 745-751.

Stephanou, A., Isenberg, D. A., Nakajima, K., and Latchman, D. S. (1999). Signal Transducer and Activator of Transcription-1 and Heat Shock Factor-1 interact and activate the transcription of the *hsp70* and *hsp90β* promoters. *J. Biol. Chem.* 274: 1723-1728.

Sterner, D. E., and Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiol. and Mol. Biol. Rev.* 64: 435-459.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms *Genes & Dev.* 12: 599-606.

Studier, F. W., Rosenberg, A. H., Lade, B. N., Chui, D. S., Lin, S. W., and Dunn, J. J. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56: 125-135.

Sullivan, S. A., Aravind, L., Makalowska, I., Baxevanis, A. D., and Landsman, D. (2000). The Histone Database: a comprehensive WWW resource for histones and histone fold-containing proteins. *Nucleic Acid Res.* 28: 320-322.

Tanabe, M., Kawazoe, Y., Takeda, S., Morimoto, R. I., Nagata, K., and Nakai, A. (1998). Disruption of the *hsf3* gene results in the severe reduction of heat shock gene expression and loss of thermotolerance. *EMBO J.* 17: 1750-1758.

Tanikawa, J., Ichikawa-Iwata, E., Kanei-Ishii, C., Nakai., A., Matsuzawa, S., Reed, J. C., and Ishii, S. (2000). p53 suppresses the c-Myb-induced activation of heat shock transcription factor 3. *J. Biol. Chem.* 275: 15578-15585.

Taylor, I. C., Workman, J. L., Schuetz, T. J., and Kingston, R. E. (1991). Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: differential function of DNA-binding domains. *Genes & Dev.* 5: 1285-1298.

Tao, Y., Strelkov, S. V., Mesyanzhinov, V. V., and Rossmann, M. G. (1997). Structure of bacteriophage T4 fibritin: a segment coiled coil and the role of the C-terminal domain. *Curr. Biol.* 5: 789-798.

Topol, J., Ruden, D. M., and Parker, C. S. (1985). Sequences required for *in vitro* transcriptional activation of a *Drosophila hsp 70* gene. *Cell* 42: 527-537.

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

Tsukiyama, T., Becker, P., and Wu, C. (1994). ATP-dependent nucleosome disruption at a heat shock promoter mediated by binding of GAGA transcription factor. *Nature* 367: 525- 532.

Venturi, B. C., Erkine, A. M., and Gross, D. (2000). Cell cycle-dependent binding of yeast heat shock factor to nucleosome. *Mol. Cell. Biol.* 20: 6435-6448.

Vierling, E. (1991). The role of heat shock proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42: 579-620.

Vignali, M., Hassan, A. H., Neely, K. E., and Workman, J. L. (2000). ATP-dependent chromatin-remodeling complexes. *Mol. Cell. Biol.* 20: 1899-1910.

Vuister, G. W., Kim, S.-J., Orosz, A., Marquardt, J., Wu, C., and Bax, A. (1994). Solution structure of the DNA-binding domain of *Drosophila* heat shock transcription factor. *Nat. Struct. Biol.* 1: 605-614.

Wang, Y., and Morgan, W. D. (1994). Cooperative interaction of human HSF1 heat shock transcription factor with promoter DNA. *Nucleic Acids Res.* 22: 3113-3118.

Waters, E. R., Lee, G. J., and Vierling, E. (1996). Evolution, structure and function of the small heat shock proteins in plants. *J. Exp. Botany* 47: 325-338.

Westwood, J. T., Clos, J., and Wu, C. (1991). Stress-induced oligomerization and chromosomal relocalization of heat shock factor. *Nature* 353: 822-827.

Westwood, J. T., and Wu, C. (1993). Activation of *Drosophila* heat shock factor: conformational change associated with a monomer-to-trimer transition. *Mol. Cell. Biol.* 13: 3481-3486.

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

Wisniewski, J., Orosz, A., Allada, R., and Wu., C. (1996). The C-terminal region of *Drosophila* heat shock factor (HSF) contains a constitutively functional transactivation domain. *Nucleic. Acids Res.* 24: 367-374.

Workman, J. L., and Kingston, R. E. (1998). Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.* 76: 545-579.

Wu, C. (1995). Heat stress transcription factors. *Annu. Rev. Cell Biol.* 11: 441-469.

Xia, Y., Hwang, L. Y., Cobb, M. H., and Baer, R. (1994). Products of the *tal2* oncogene in leukemia T cells: bHLH phosphoproteins with DNA-binding activity. *Oncogene* 9: 1437-1446.

Xia, W., and Voellmy, R. (1997). Hyperphosphorylation of heat shock transcription factor 1 is correlated with transcriptional competence and slow dissociation of active factor trimers. *J. Biol. Chem.* 272: 4094-4102.

Xia, W., Guo, Y., Vilaboa, N., Zuo, J., and Voellmy, R. (1998). Transcriptional activation of heat shock transcription factor HSF1 probed by phosphopeptide analysis of factor ^{32}P-labeled *in vivo*. *J. Biol. Chem.* 273: 8749-8755.

Xiao, H., and Lis, J. T. (1988). Germline transformation used to define key features of heat-shock response elements. *Science* 4: 1139-1142.

Xiao, H., Perisic, O., and Lis, J. T. (1991). Cooperative binding of *Drosophila* heat shock factor to arrays of a conserved 5 bp unit. *Cell* 64: 585–593.

Xiao, X. Z., Zuo, X. X., Davis, A. A., McMillan, D. R., Curry, B. B., Richardson, J. A., and Benjamin, I. J. (1999). HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO J.* 18: 5943-5952.

Yoshima,T., Yura, T., and Yanagi, H. (1997). The trimerization domain of human heat shock factor 2 is able to interact with nucleoporin p62. *Biochem. Biophys. Res. Commun.* 240: 228-233.

Yoshima,T., Yura, T., and Yanagi, H. (1998). Novel testis-specific protein that interacts with heat shock factor 2. *Gene* 214: 139-146.

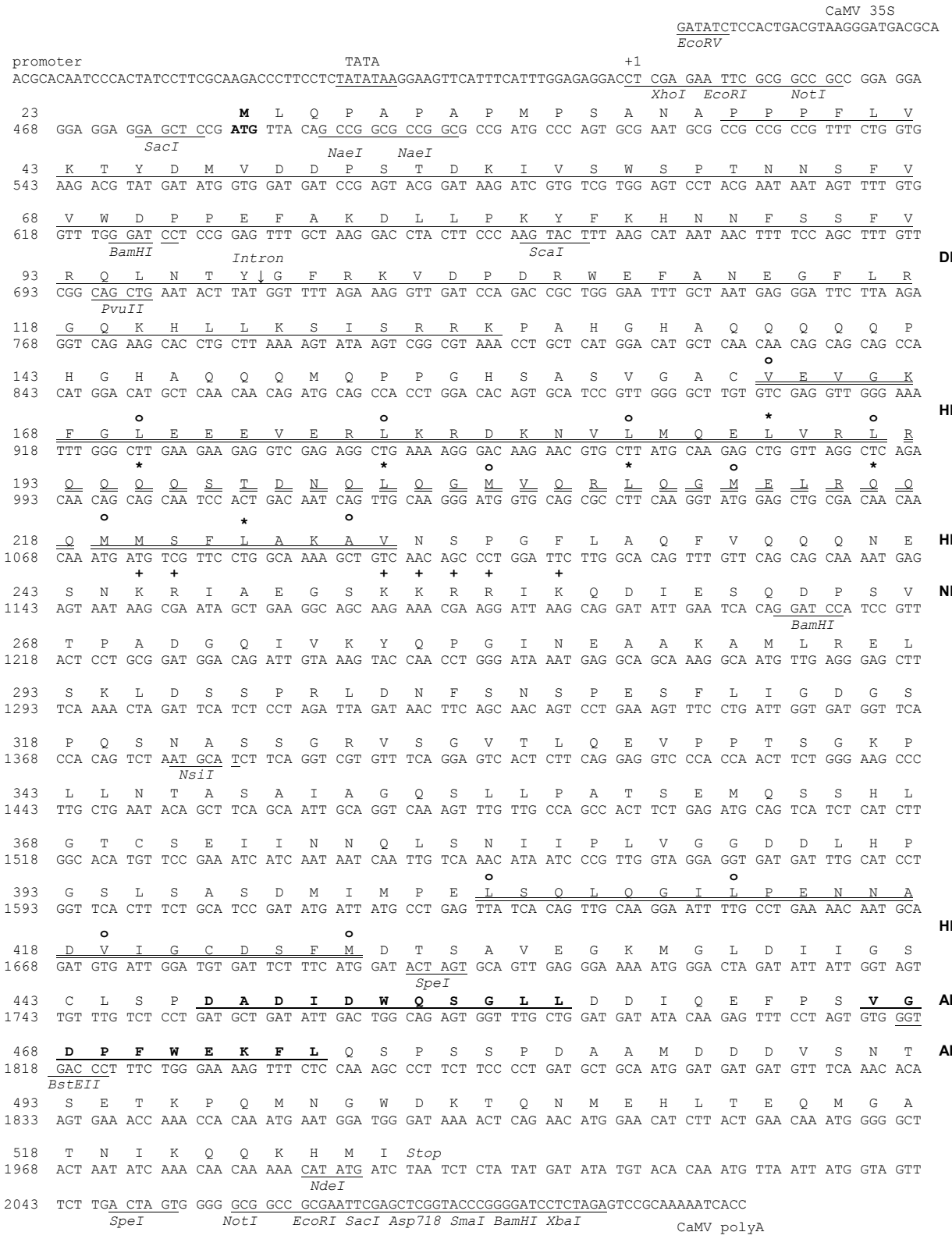
Zhu, B. Y., Zhou, N. E., Kay, C. M. and Hodges, R. S. (1993). Packing and hydrophobicity effects on protein folding and stability: effects of beta-branched amino acids, valine and isoleucine, on the formation and stability of two-stranded alpha-helical coiled coils/leucine zippers. *Protein Sci.* 2: 383-394.

Zuo, J., Baler, R., Dahl, G., and Voellmy, R. (1994). Activation of the DNA-binding ability of human heat shock transcription factor 1 may involve the transition from an intramolecular to an intermolecular triple-stranded coiled-coil structure. *Mol. Cell. Biol.* 14: 7557-7568.

Zuo, J., Rungger, D., and Voellmy, R. (1995). Multiple layers of regulation of human heat shock transcription factor 1. *Mol. Cell. Biol.* 15: 4319-4330.

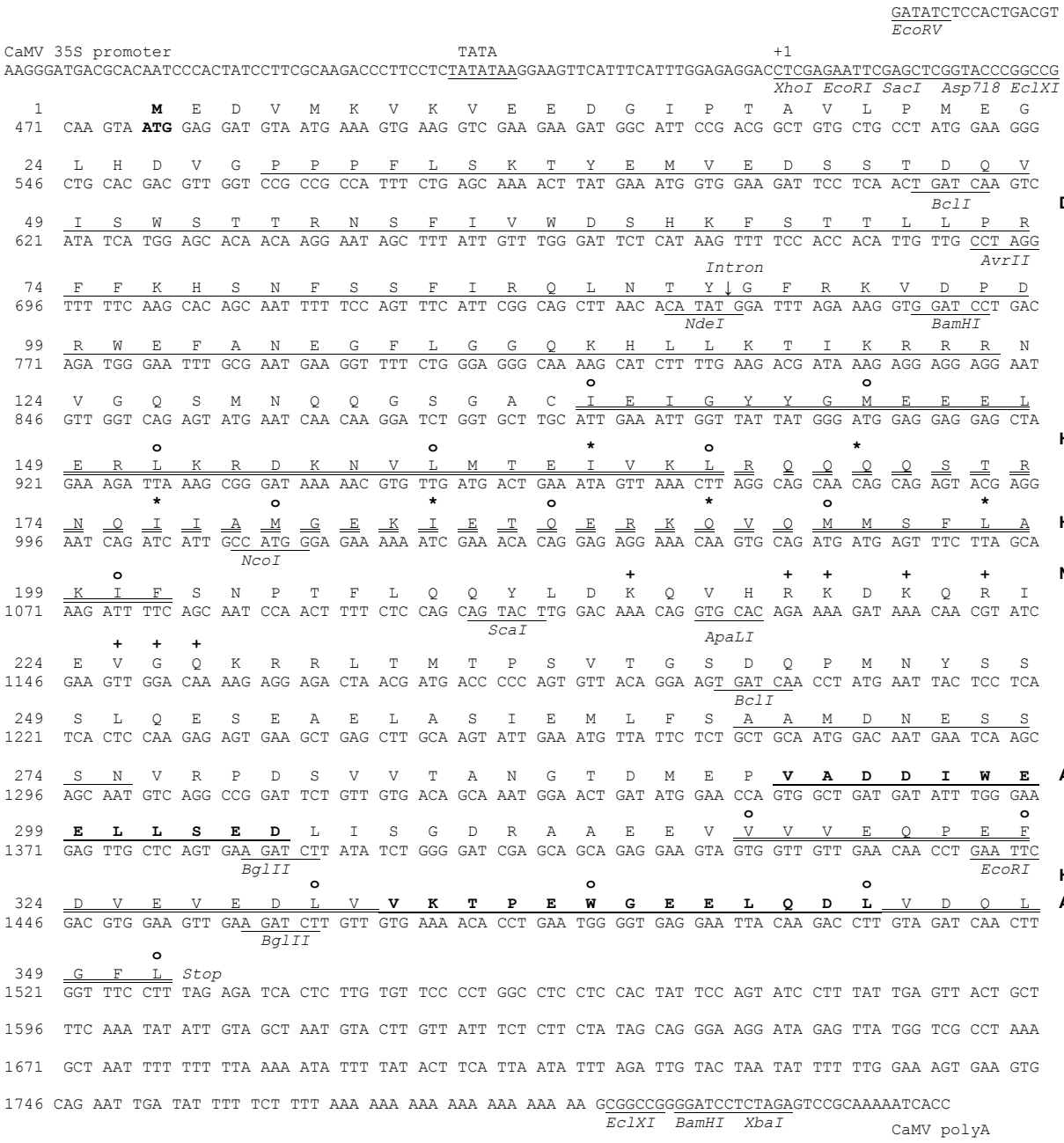
Zou, Z., Guo, Y., Guettouche, T., Smith, D., and Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by hsp90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94: 471-480.

Appendix A: Protein and c-DNA sequences of Hsfs and sHsps

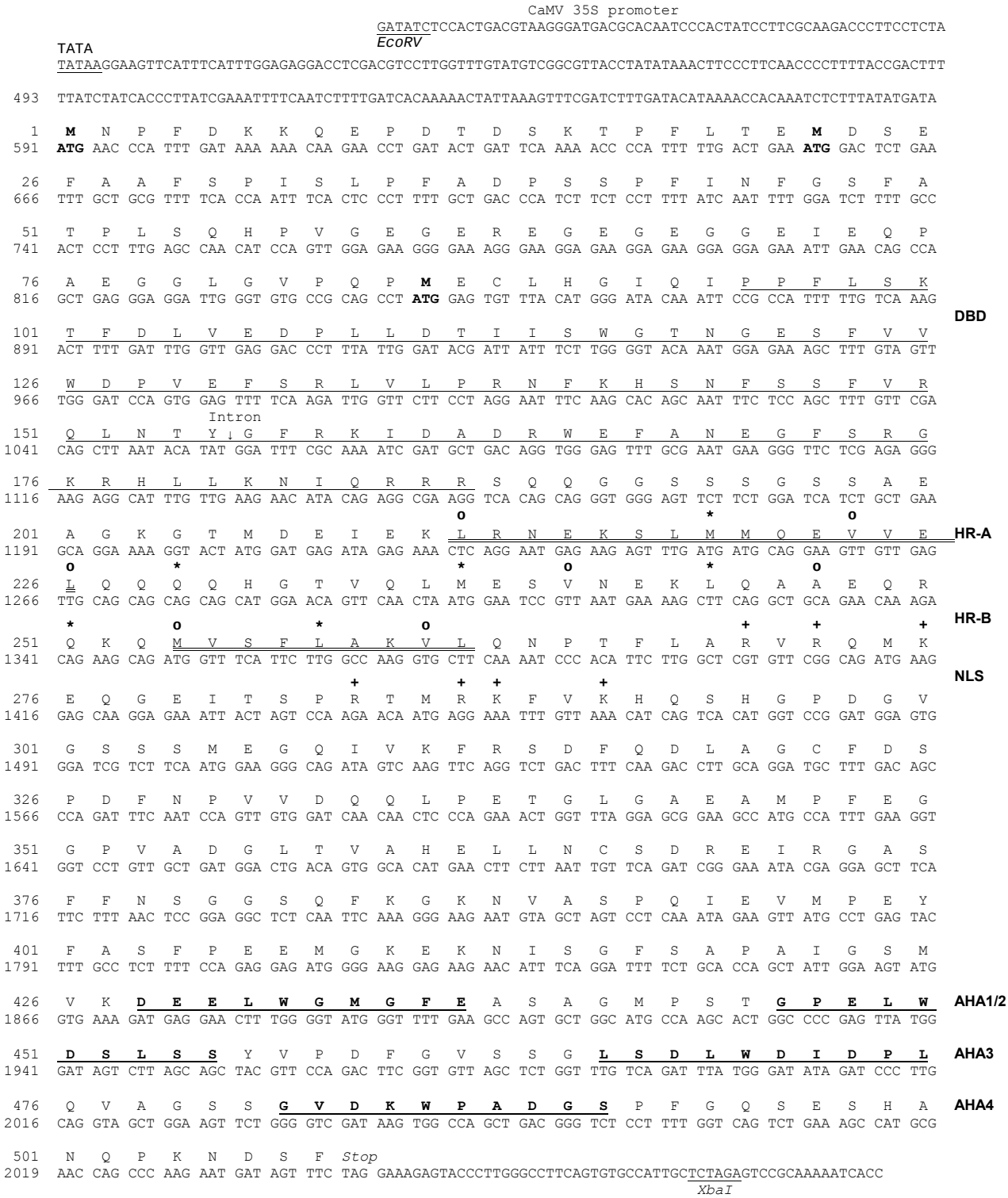


Protein and c-DNA sequence of tomato HsfA1

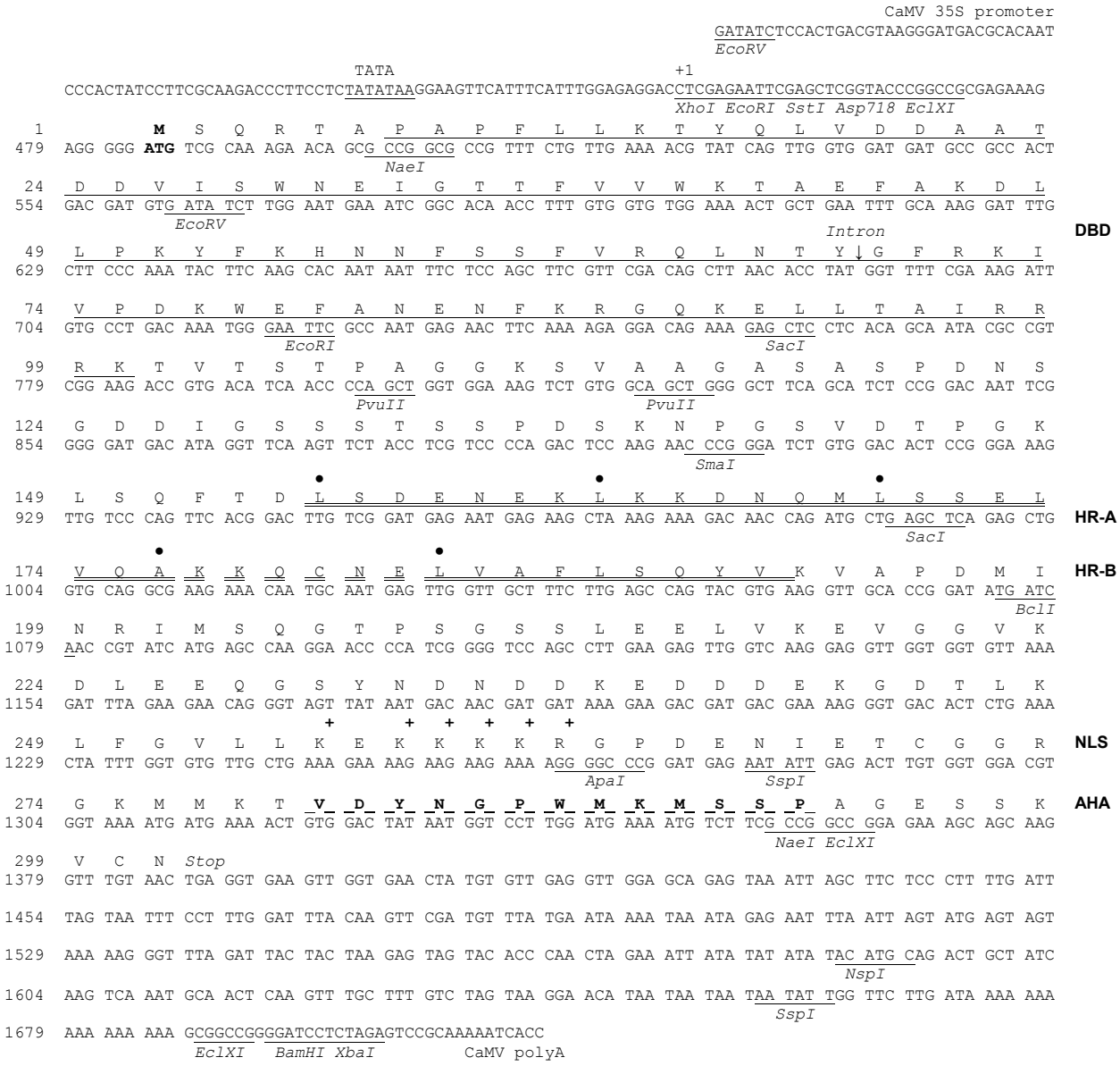
Original name: Lp-Hsf8 [*Lycopersicon peruvianum*]
Database accession number: CAA47868



Protein and c-DNA sequence of tomato HsfA2
Original name: Lp-Hsf30 [*Lycopersicon peruvianum*]
Database accession number: X67601



Protein and c-DNA sequence of tomato HsfA3
Original name: Lp-HsfA3 [*Lycopersicon peruvianum*]
Database accession number: AF208544



Protein and c-DNA sequence of tomato HsfB1
Original name: Lp-Hsf24 [*Lycopersicon peruvianum*]
Database accession number: CAA39034

```
  1            M   G   L   I   P   R   I   F   G   D   R   R   S   S   S   M
939   GAA TTC ATG GGT CTG ATC CCA AGA ATC TTC GGC GAC CGA CGA AGC AGT AGC ATG
      EcoRI
 19    F   D   P   F   S   I   D   V   F   D   P   F   R   E   L   G   F   P
993   TTC GAT CCA TTT TCA ATT GAC GTA TTT GAT CCA TTT AGG GAA TTA GGC TTT CCA

 35   G   T   N   S   R   E   T   S   A   F   A   N   T   R   I   D   W   K
1047  GGT ACC AAT TCA CGG GAG ACC TCT GCA TTT GCT AAC ACT CGA ATC GAT TGG AAG

 53   E   T   P   E   A   H   V   F   K   A   D   L   P   G   L   K   K   E
1101  GAA ACT CCA GAA GCT CAT GTG TTC AAA GCC GAT CTT CCA GGG CTT AAG AAG GAG

 71   E   V   K   V   E   I   E   E   D   R   V   L   Q   I   S   G   E   R
1155  GAA GTG AAA GTG GAG ATC GAA GAA GAT AGG GTT CTT CAG ATC AGC GGA GAG AGG

 89   N   V   E   K   E   D   K   N   D   T   W   H   R   V   E   R   G   S
1209  AAC GTG GAG AAA GAA GAT AAG AAC GAT ACT TGG CAT CGT GTG GAA CGC GGC AGT

107   G   K   F   M   R   R   F   R   L   P   E   N   A   K   M   D   Q   V
1263  GGA AAA TTC ATG AGG AGA TTT AGA CTT CCG GAG AAC GCA AAG ATG GAT CAA GTT

125   K   A   S   M   E   N   G   V   L   T   V   T   V   P   K   E   E   V
1317  AAG GCT TCT ATG GAG AAT GGA GTG CTG ACT GTC ACT GTT CCA AAG GAA GAA GTG

143   K   K   P   D   V   K   S   I   E   I   S   G  Stop
1371  AAG AAG CCT GAT GTC AAG TCT ATT GAA ATC TCT GGT TAA GTC GAC TCG AG
                                                        XhoI
```

Protein and c-DNA sequence of tomato Hsp17.6(I)
Original name: class I Lp-Hsp17.6 [*Lycopersicon peruvianum*]
New name: class CI Lp-Hsp17.6 [*Lycopersicon peruvianum*] (Scharf et al., 2001)
Reference: It has been recently described by Löw et al. (2000)

```
939                                                GAA TTC GGC ACG AGC AAA CAC GAA
                                                    EcoRI
  1           M   D   L   R   L   M   G   I   D   N   T   P   L   F   H   T
 963  AGA AGA ATG GAT TTG AGG TTG ATG GGT ATC GAT AAC ACA CCA CTT TTC CAC ACT

 19   L   Q   H   M   M   E   A   A   G   E   D   S   V   N   A   P   S   K
1017  CTT CAG CAT ATG ATG GAA GCT GCC GGT GAA GAT TCG GTG AAT GCA CCA TCA AAG

 35   K   Y   V   R   D   A   K   A   M   A   A   T   P   V   D   V   K   E
1071  AAG TAT GTT CGT GAT GCT AAG GCA ATG GCT GCG ACA CCA GTG GAC GTG AAA GAG

 53   Y   P   D   S   Y   V   F   V   V   D   M   P   G   L   K   S   G   D
1125  TAT CCT GAT TCA TAT GTT TTC GTT GTG GAT ATG CCA GGG TTG AAA TCT GGA GAT

 71   I   N   V   Q   V   E   E   D   N   V   L   L   I   S   G   E   R   K
1179  ATC AAT GTG CAG GTA GAA GAA GAC AAT GTG CTG TTG ATT AGT GGT GAA AGG AAG

 89   R   E   E   E   K   E   G   V   K   F   I   R   M   E   R   R   V   G
1233  AGG GAA GAA GAG AAA GAA GGT GTA AAG TTT ATT AGA ATG GAG AGA AGG GTT GGG

107   K   F   M   R   K   F   S   L   P   E   N   A   N   T   D   A   I   S
1287  AAA TTC ATG AGG AAG TTT AGT CTG CCG GAG AAT GCG AAT ACT GAT GCA ATT TCT

125   A   V   C   Q   D   G   V   L   T   V   T   V   R   K   L   P   P   P
1341  GCA GTT TGT CAA GAT GGA GTT CTG ACT GTT ACT GTT CGG AAG CTG CCT CCT CCT

143   E   P   K   K   P   K   T   I   Q   V   K   V   A  Stop
1395  GAG CCA AAG AAG CCC AAA ACC ATT CAG GTC AAA GTC GCT TGA AAA TAT AAA GTC

1449  ACT CTG TTT TCT TGC TCT GTT TTG ATG TAA TGA AGT GAT TTA CCA TAT TTT GAT

1503  GCA TCC AAG GAT TAA GAA AAT ACA AAT TTT AGT GGA TGT ATC TTG TTT GAT AAA

1557  GAA TCG AAT TTT AAT TAC TTT TTG CCT CAA AAA AAA AAA AAA AAA AAA AAA AAA

1611  AAA AAA AAA AAA AAA AAA AAA ACT CGA G
                                    XhoI
```

Protein and c-DNA sequence of tomato Hsp17(II)

Original name: class II Lp-Hsp17 type I, clone 76.1 [*Lycopersicon peruvianum*]

New name: class CII Lp-Hsp17 type I, clone 76.1 [*Lycopersicon peruvianum*] (Scharf et al., 2001)

Reference: It has been recently identified in our laboratory (Chen A., diploma work,1999)

```
 939                                                                GAA TTC GGC
                                                                    EcoRI
   1          V   S   Q   L   L   F   P   E   S   I   E   R   L   V   S   P
 948  ACG AGA GTG AGC CAA CTT CTC TTT CCA GAA TCC ATT GAG AGG CTG GTG AGT CCT

  19   S   R   S   N   E   S   K   G   T   I   P   V   D   I   L   D   T   P
1002  TCT AGG TCA AAT GAG AGT AAG GGT ACC ATT CCC GTG GAC ATT CTG GAT ACC CCA

  35   K   E   Y   I   F   Y   M   D   V   P   G   L   S   K   S   D   L   Q
1056  AAA GAG TAC ATT TTT TAT ATG GAT GTT CCT GGT TTA TCC AAA TCT GAC CTA CAG

  53   V   S   V   E   D   E   K   T   L   V   I   R   S   N   G   K   R   K
1110  GTG AGT GTG GAA GAT GAG AAG ACA CTG GTG ATA CGA AGC AAC GGG AAG AGG AAG

  71   R   E   E   S   E   E   E   G   C   K   Y   V   R   L   E   R   N   P
1164  CGC GAG GAG AGT GAA GAA GAG GGA TGC AAG TAC GTG AGG TTG GAG AGG AAT CCA

  89   P   L   K   L   M   R   K   F   K   L   P   D   Y   C   N   V   S   A
1218  CCC CTC AAA CTG ATG AGG AAG TTC AAG CTG CCC GAT TAC TGC AAT GTT TCT GCT

 107   I   T   A   K   C   E   N   G   V   L   T   V   V   V   E   K   M   P
1272  ATT ACT GCC AAA TGC GAG AAT GGG GTG TTA ACT GTT GTA GTT GAA AAG ATG CCT

 125   P   P   S   K   A   K   T   V   K   V   A   V   S  Stop
1326  CCA CCA TCT AAG GCG AAG ACT GTT AAA GTT GCA GTT TCA TGA ATT AAA TTA TCA


1380  TAA ATC TAC ATC TCT CTT ACT TCA GCT TTT ATT AAT TAT ATC TTA CTT TTA CAA


1434  AGA ACG GCC TTT TAC TCC TTA TGT TAA GGC TAA TTT CTT ATT TTA ATT CTT GNG


1488  GTA TTT AAC TTA NTA TTA TTA ATT TTA ATT AAA AAA AAA AAA AAA AAA ACTCGAG
                                                                    XhoI
```

Protein and c-DNA sequence of tomato sHsp(IIs)
Original name: special class II Lp-Hsp17, clone 8.2 [*Lycopersicon peruvianum*]
New name: class CIII Lp-Hsp17, clone 8.2 [*Lycopersicon peruvianum*] (Scharf et al., 2001)
Reference: It has been recently identified in our laboratory (1999)

```
   1                M   S   L   I   P   S   F   F   S   G   R   R   S   N   V   F
 857  GAA TTC ATG TCT CTG ATT CCA AGT TTC TTT AGT GGC CGA AGG AGC AAT GTT TTC
       EcoRI
  19   D   P   F   S   R   D   V   W   D   P   L   K   D   F   P   F   S   N
 911  GAT CCT TTC TCC CGG GAC GTC TGG GAT CCT TTG AAG GAC TTT CCA TTT TCA AAT

  35   S   S   L   S   A   S   ?   P   R   E   N   P   A   F   V   S   T   R
 965  TCT TCA CTT TCC GCT TCA TNC CCT CGT GAG AAT CCT GCT TTT GTG AGC ACA CGA

  53   V   D   W   K   E   T   P   E   A   H   V   F   K   A   D   L   P   G
1019  GTT GAC TGG AAG GAA ACA CCG GAA GCG CAT GTT TTC AAG GCT GAT CTT CCT GGG

  71   L   K   K   E   E   V   K   V   E   V   E   D   D   R   V   L   Q   I
1073  CTG AAA AAG GAG GAA GTG AAA GTT GAA GTT GAA GAT GAT AGG GTT CTA CAG ATA

  89   S   G   E   R   S   V   E   K   E   D   K   N   D   E   W   H   R   V
1127  AGC GGA GAG AGA AGC GTT GAG AAA GAA GAT AAG AAT GAT GAA TGG CAT CGC GTG

 107   E   R   S   S   G   K   F   L   R   R   F   R   L   P   E   N   A   K
1181  GAA CGT AGC AGT GGA AAG TTC TTA AGA AGG TTC AGA TTG CCT GAG AAT GCT AAA

 125   M   D   K   V   K   A   S   M   E   N   G   V   L   T   V   T   V   P
1235  ATG GAT AAA GTG AAA GCT TCC ATG GAG AAC GGC GTT CTG ACA GTG ACC GTT CCA

 143   K   E   E   I   K   K   A   E   V   K   S   I   E   I   S   G  Stop
1289  AAA GAA GAG ATA AAG AAG GCT GAG GTT AAG TCT ATT GAG ATT TCT GGT TAA ACT

1343  TAG TCG AC
       Sal I
```

Protein and c-DNA sequence of pea Hsp18.1(I)
Original name: class I Ps-Hsp18.1 [*Pisum sativum*]
New name: class CI Ps-Hsp18.1 [*Pisum sativum*] (Scharf et al., 2001)
Database accession number: AAA33672

```
 936                                                        G AAT TCA ATC GAT TTC
                                                            EcoRI
   1           M   D   L   D   S   P   L   F   N   T   L   H   H   I   M
 954  AGG CTA ATG GAT TTG GAT TCT CCA CTC TTC AAC ACT CTC CAT CAT ATA ATG

  19   D   L   T   D   D   T   T   E   K   N   L   N   A   P   T   R   T
1005  GAC CTC ACC GAC GAC ACA ACC GAG AAG AAC TTA AAC GCT CCA ACT CGA ACA

  35   Y   V   R   D   A   K   A   M   A   A   T   P   A   D   V   K   E
1056  TAT GTC CGT GAC GCA AAG GCA ATG GCT GCA ACT CCA GCG GAC GTG AAA GAG

  53   H   P   N   S   Y   V   F   M   V   D   M   P   G   V   K   S   G
1107  CAT CCA AAT TCA TAC GTG TTT ATG GTG GAC ATG CCT GGG GTG AAA TCT GGT

  71   D   I   K   V   Q   V   E   D   E   N   V   L   L   I   S   G   E
1158  GAC ATA AAG GTT CAG GTG GAA GAT GAG AAT GTG CTA TTG ATA AGT GGC GAG

  89   R   K   R   E   E   E   K   E   G   V   K   Y   L   K   M   E   R
1209  AGG AAG AGA GAA GAA GAG AAA GAA GGT GTT AAA TAT TTG AAG ATG GAA AGA

 107   R   I   G   K   L   M   R   K   F   V   L   P   E   N   A   N   I
1260  AGG ATT GGT AAG TTG ATG AGG AAA TTT GTG TTA CCT GAG AAT GCG AAT ATT

 125   E   A   I   S   A   I   S   Q   D   G   V   L   T   V   T   V   N
1311  GAA GCT ATC TCT GCT ATT TCT CAA GAT GGT GTT CTT ACG GTT ACA GTT AAT

 143   K   L   P   P   P   E   P   K   K   P   K   T   I   Q   V   K   V
1362  AAA TTG CCT CCA CCT GAA CCT AAG AAA CCA AAA ACT ATT CAA GTT AAG GTT

 160   A  Stop
1413   GCT TGA TCG GTG TCT CGA G
                       XhoI
```

Protein and c-DNA sequence of pea Hsp17.7(II)
Original name: class II Ps-Hsp17.7 [*Pisum sativum*]
New name: class CII Ps-Hsp17.7 [*Pisum sativum*] (Scharf et al., 2001)
Database accession number: AAA33670

```
 938            G AAT TCG GCA CGA GAT CAA AGA ATC AGG AAA GCA AAC ACA ACA CAA
                 EcoRI
   1            M   D   L   G   R   F   P   I   I   S   I   L   E   D   M   L
 984  CTA ACA ATG GAT TTA GGA AGG TTT CCA ATA ATC TCA ATC CTC GAA GAC ATG CTT

  19   E   V   P   E   D   H   N   N   E   K   T   R   N   N   P   S   R   V
1038  GAA GTC CCC GAA GAC CAC AAC AAC GAG AAG ACC CGC AAC AAC CCT TCA CGA GTT

  35   Y   M   R   D   A   K   A   M   A   A   T   P   A   D   V   I   E   H
1092  TAC ATG CGA GAC GCT AAG GCA ATG GCT GCT ACA CCT GCT GAC GTC ATC GAG CAC

  53   P   N   A   Y   A   F   V   V   D   M   P   G   I   K   G   D   E   I
1146  CCT AAC GCA TAT GCA TTC GTC GTG GAC ATG CCT GGA ATC AAA GGA GAT GAG ATC

  71   K   V   Q   V   E   N   D   N   V   L   V   V   S   G   E   R   Q   R
1200  AAG GTT CAG GTC GAG AAC GAC AAT GTG CTT GTG GTG AGT GGA GAA AGG CAG AGA

  89   E   N   K   E   N   E   G   V   K   Y   V   R   M   E   R   R   M   G
1254  GAG AAC AAA GAA AAC GAA GGT GTG AAG TAT GTG AGG ATG GAG AGA AGA ATG GGT

 107   K   F   M   R   K   F   Q   L   P   E   N   A   D   L   D   K   I   S
1308  AAG TTC ATG AGG AAG TTT CAG TTG CCT GAA AAT GCA GAT TTG GAC AAG ATC TCT

 125   A   V   C   H   D   G   V   L   K   V   T   V   Q   K   L   P   P   P
1362  GCT GTT TGT CAT GAC GGT GTG TTG AAG GTT ACT GTT CAG AAA CTT CCT CCT CCG

 143   E   P   K   K   P   K   T   I   Q   V   Q   V   A  Stop
1416  GAA CCA AAG AAA CCA AAG ACA ATT CAA GTT CAA GTT GCT TGA GTT TGT TTT TCT


1470  AGG GCC CGA GCT CGA ATT CCC GGG TCG ACT CGA G
                                          XhoI
```

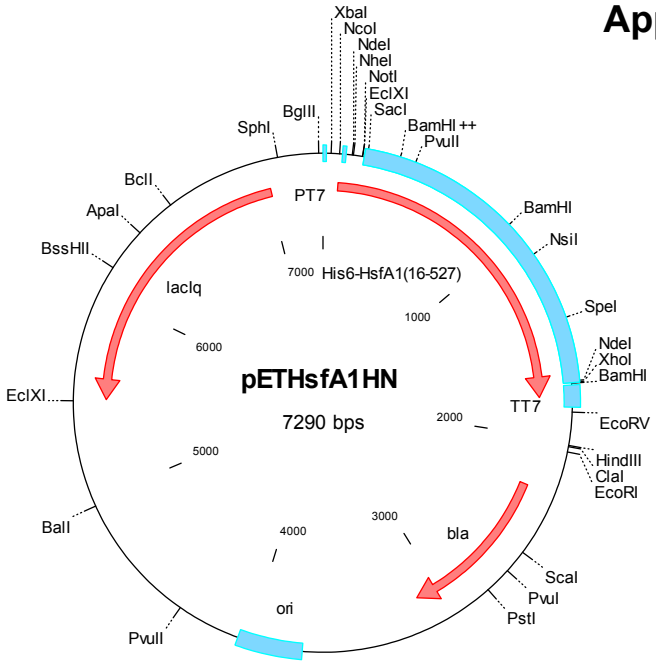
Protein and c-DNA sequence of *Arabidopsis* Hsp17.6(II)
Original name: class II At-Hsp17.6 [*Arabidopsis thaliana*]
New name: class CII At-Hsp17.6 [*Arabidopsis thaliana*] (Scharf et al., 2001)
Database accession number: AAA33672

Appendix B: Oligonucleotides list

Code N.	Sequence	Priming region	USE
77	5´- cacagatagattggcttc-3`	pBD Gal4 N-terminus	Sequencing and PCR
78	5´- gaccaaactgcgtataacg-3`	pBD Gal4 C-terminus	Sequencing and PCR
141	5´- cagttgaagtgaacttgcggg-3´	pBD Gal4 N-terminus	Sequencing and PCR
142	5´- cctacaggaaagagttactcaagaac-3`	pBD Gal4 C-terminus	Sequencing and PCR
309	5´- gctaagaagctagccgcctgcacttgt ttcctctcc-3´	HR-B region of HsfA2	PCR, mutation MM>AA
310	5´- gctgaaaatctttgccgcggcactcatc atctgc-3´	HR-B region of HsfA2	PCR, mutation FL>AA
311	5´- ggagaaaagttggattgctagcagcct ttgctaag-3´	HR-B region of HsfA2	PCR, mutation IF>AA
512	5´- ctagtctagagagctcgcgcgcttgctg cctaagtttaactatttcag-3´	HR-A/B linker region of HsfA2	PCR, mutation HR-A
513	5´- taattggcgcgccagatgatgagtttctt agc-3´	HR-A/B linker region of HsfA2	PCR, mutation HR-21
514	5´- ttattggcgcgcggcgcccagcagagt .....acgaggaatcagatc-3´	HR-A/B linker region of HsfA2	PCR, mutation HR+4
515	5´- ttattggcgcgcggagtcggcgcccag cagagtacgaggaatcagatcattgcc-3´	HR-A/B linker region of HsfA2	PCR, mutation HR+6
187	5´- gggctggcaagccacgtttggtg -3´	5´ MCS of pGEX vectors	Sequencing and PCR
188	5´- ccgggagctgcatgtgtcagagg-3´	3´ MCS of pGEX vectors	Sequencing and PCR
35SN1	5´- actgacgtaagggatga-3´	5´ MCS of pRT vectors	Sequencing and PCR
179	5´- gaggtcttattacacac -3´	3´ MCS of pRT vectors	Sequencing and PCR

The table reports code number (Code N.), sequence and priming region of oligonucelotides used (USE) to generate by PCR and to sequence several plasmids with mutations in the oligomerization domain of HsfA2.

Appendix C: Plasmid maps



Name in text: **His_6-HsfA1 protein (tag at the N-terminus)** Code N.:

Insert: **Lp-HsfA1 cDNA fragment** **Ni 85**

Cloning Vector: **6HisT-pET11d**

Host Organisms: Functional Markers:

1. **E. coli DH5α** **Amp^r**
2. **E. coli BL21(DE3)** **IPTG-ind. T7 RNA-Pol. expr.**

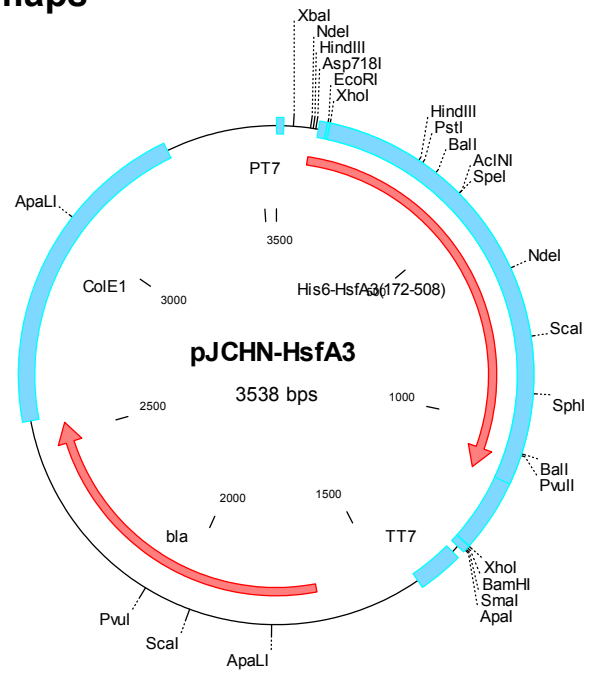
Cloning : **pET3bHsfA1/NdeI inserted into 6HisT-pET11d/NdeI-ClP**

Used for: **Expression of recombinant Lp-HsfA1 (aa 16-527,I527L) with N-term. His6-tag and thrombin cleavage site in E. coli;**

38aa (MGSSHHHHHHSSGLVPR<GSHMASMTGGQQMGRDQFAAAA) N-term. & 21aa C-term. added (total 571aa)

Constructed by/obtained from: **K.-D.Scharf** Date:

Ref.: Sequencing:



Name in text: **His_6-HsfA3 protein (tag at the N-terminus)** Code N.:

Insert: **Lp-HsfA3 cDNA fragment** **SE 3**

Cloning Vector: **pJC20HN**

Host Organisms: Functional Markers:

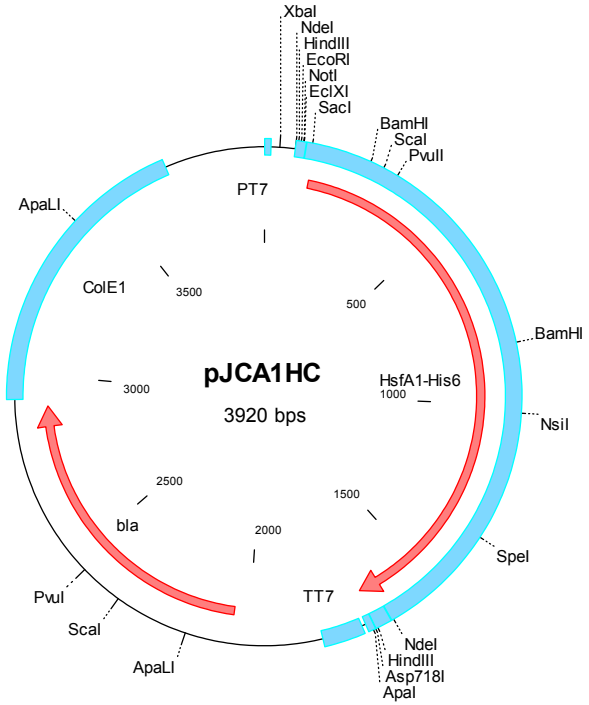
1. **E. coli DH5α** **Amp^r**
2. **E. coli BL21(DE3)** **IPTG-ind. T7 RNA-Pol. expr.**

Cloning: **Ligation of XhoI fragment of pAD103.2 into pJC20HN cut XhoI**

Used for: **Expression of recombinant Lp-HsfA3 (aa 172-508) with C-term. His6-tag and thrombin cleavage site in E. coli**

Constructed by/obtained from: **E.Schmidt/H.Listau** Date: **03/98**

Reference: **Lab. journal H. Listau** Sequencing:



Name in text: **His_6-HsfA1 protein (tag at the C-terminus)** Code N.:

Insert: **Lp-HsfA1 cDNA** **Ni 206**

Cloning Vector: **pJC20**

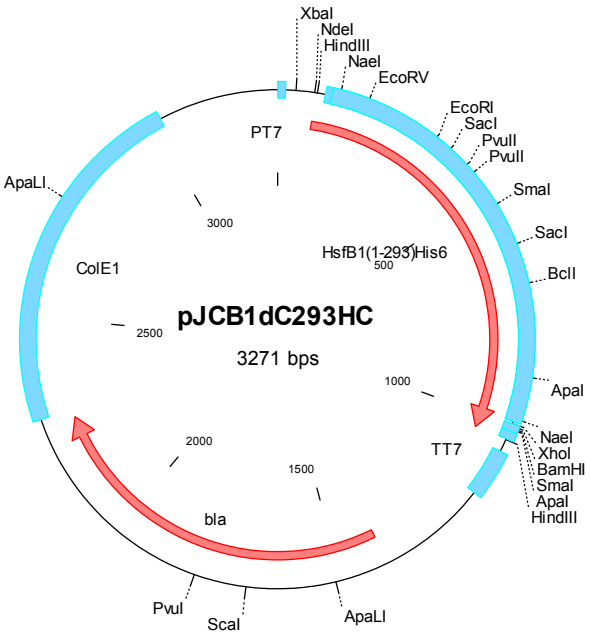
Host Organisms:	Functional Markers:
1. **E. coli DH5α**	**Amp^r**
2. **E. coli BL21(DE3)**	**IPTG-ind. T7 RNA-Pol. expr.**
3. **E. coli BL21(DE3), pLysS**	**+Cap^r, lysozyme expression**

Cloning : **pRTHsfA1PHC/EcoRI-ApaI inserted into pJC20/EcoRI-ApaI**

Used for: **Expression of recombinant Lp-HsfA1 (aa 23-527,I527<L) with C-term. His6-tag and thrombin cleavage site in E. coli; 15aa N-term. (MKLEFAAAGGGGGAP) & 11aa(VPRGSHHHHHH) C-term. added**

Constructed by/obtained from: **K.-D.Scharf** Date:

Ref: Sequencing: **WS102**



Name in text: **His_6-HsfB1 protein (tag at the C-terminus)** Code N.:

Insert: **Lp-HsfB1 cDNA fragment** **Ni 16**

Cloning Vector: **pJC20HC**

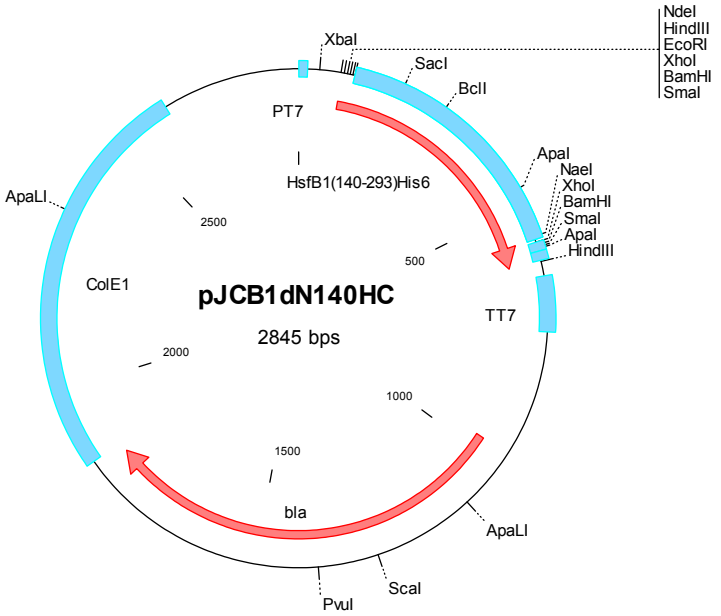
Host Organisms:	Functional Markers:
1. **E. coli DH5α**	**Amp^r**
2. **E. coli BL21(DE3)**	**IPTG-ind. T7 RNA-Pol. expr.**
3. **E. coli BL21(DE3), pLysS**	**+Cap^r, lysozyme expression**

Cloning : **pRTHsfB1/EclXI-Klenow inserted into pJC20HC/EcoRI-Klenow-CIP**

Used for: **Expression of recombinant Lp-HsfB1 (aa 1-293) with C-term. His6-tag in E. coli, 11aa (MKLELAARKRG) N-term. & 13aa (NSRGSRAHHHHHH) C-term. added, (total 317aa)**

Constructed by/obtained from: **K.-D.Scharf** Date:

Ref.: Sequencing:



Name in text: **His_6-HsfB1 CTD protein (tag at the C-terminus)** Code N.:

Insert: **Lp-HsfB1 dN140 cDNA fragment** **RC43**

Cloning Vector: **pJC20HC**

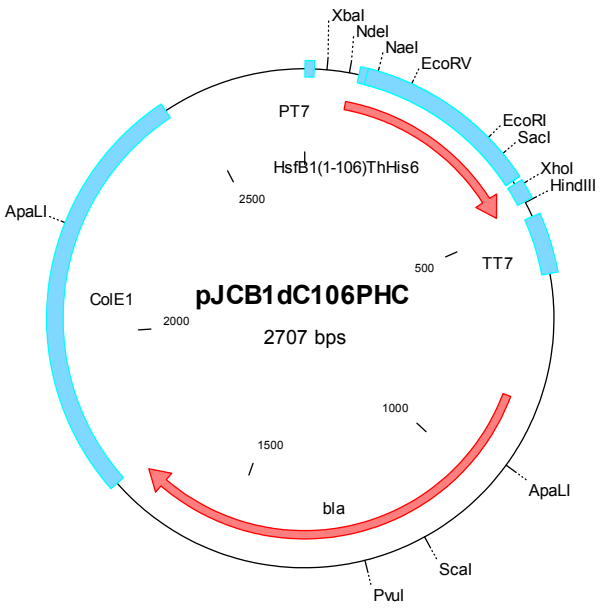
Host Organisms:	Functional Markers:
1. **E. coli DH5α**	**Amp^r**
2. **E. coli BL21(DE3)**	**IPTG-ind. T7 RNA-Pol. expr.**
3. **E. coli BL21(DE3), pLysS**	**+Cap^r, lysozyme expression**

Cloning: **pJCB1dC292HC/SmaI inserted into pJC20HC/SmaI**

Used for: **Expression of recombinant Lp-HsfB1 (aa 140-293) with C-term. His6-tag in E. coli, 11aa (MKLEFSRI) N-term. & 13aa (NSRGSRAHHHHHH) C-term. added, (total 175 aa)**

Constructed by/obtained from: **R. Calligaris** Date: **06/99**

Reference: **Laborbuch, Promotion** Sequencing: **RC79/RC80**



Name in text: **His_6-HsfB1 DBD protein (tag at the C-terminus)** Code N.:

Insert: **Lp-HsfB1 cDNA fragm.** **F 1028**

Cloning Vector: **pJC20**

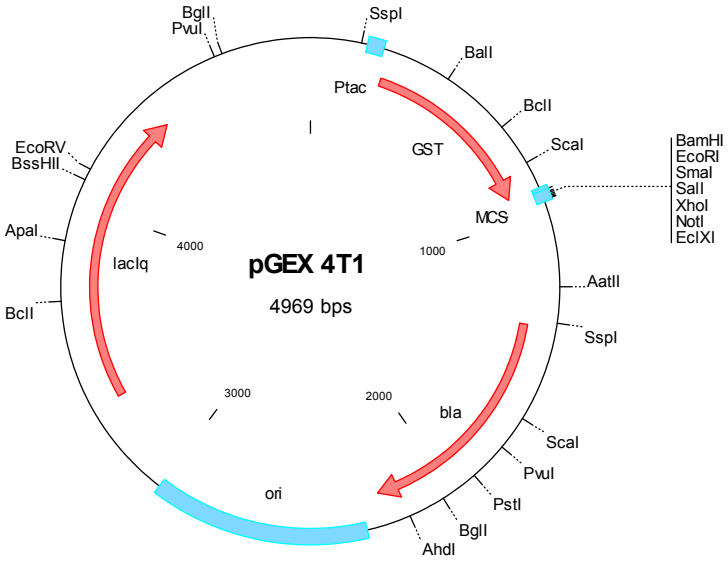
Host Organisms:	Functional Markers:
1. **E. coli DH5α**	**Amp^r**
2. **E. coli BL21(DE3)**	**IPTG-ind. T7 RNA-Pol. expr.**
3. **E. coli BL21(DE3), pLysS**	**+Cap^r, lysozyme expression**

Cloning : **Insertion of linker LiPRO1/2 into pJCB1dC106HC/ApaI-HindIII**

Used for: **Expression of recomb. Lp-HsfB1 (aa 1-106) with C-term. His6-tag & Thrombin cleavage site, 9aa (MKLAARKRG) N-term. & 14aa C-term. (GALVPR>GSHHHHHH) added**

Constructed by/obtained from: **K.-D.Scharf** Date:

Reference: **Schultheiss et al. EJB 236(96)911** Sequencing:



Name in text: **GST protein** Code N.:

Insert: **RC20**

Cloning Vector: **pGEX 4T1**

Host Organisms: Functional Markers:

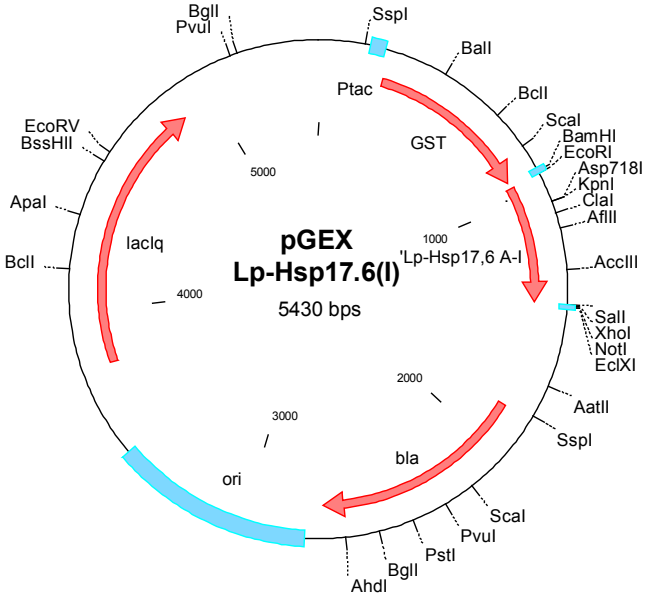
1. **E. coli DH5α** **Amp^r;**
2. **E. coli BL21(DE3)** **GSTxTCSxMCS**

Used for: **Expression of GSTxTCSxfusion proteins in bacteria**

Remarks:

Constructed by/obtained from: **Pharmacia** Date:

Reference: Sequencing:



Name in text: **GST Lp-Hsp17.6(I) protein** Code N.:

Insert **Lp-Hsp17.6 class I** **RC 48**

Cloning Vector: **pGEX 4T1**

Host Organisms: Functional Markers:

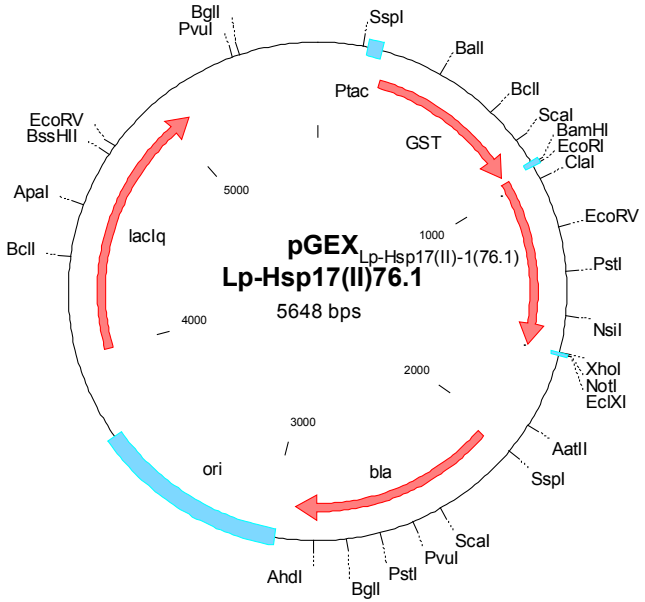
1. **E. coli DH5α** **Amp^r;**
2. **E. coli BL21(DE3)** **GSTxTCSxLp-Hsp17.6(I)**

Cloning: **Ligation of EcoRI/SalI PCR amplified fragment (Pr.409F/410R) of pRTLp-Hsp17.6(I) into EcoRI/SalI cut pGEX 4T1**

Used for: **Expression of GSTxTCSxLp-Hsp17.6(I) (aa1-154) in bacteria**

Constructed by/obtained from: **R. Calligaris** Date: **09/99**

Reference: **Laborbuch, Promotion** Sequencing: **RC100**



Name in text: **GST Lp-Hsp17(II) protein** Code N.:

Insert **Lp-Hsp17 class II, type1, clone 76.1** **RC 44**

Cloning Vector: **pGEX 4T1**

Host Organisms: Functional Markers:

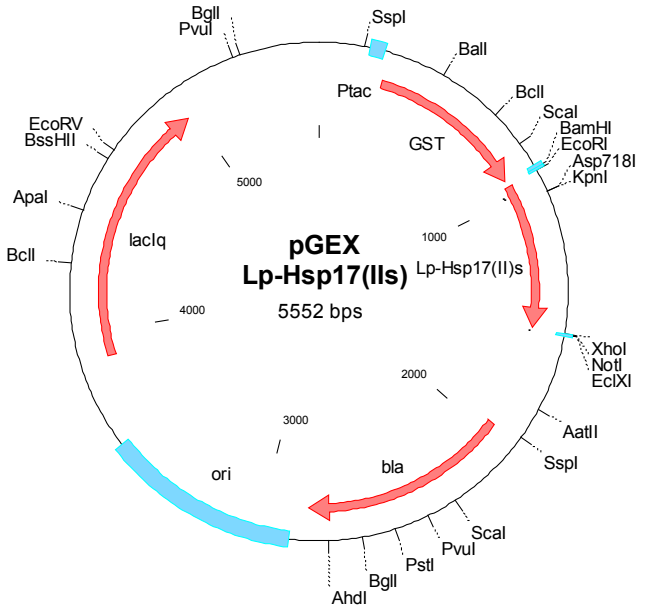
1. **E. coli DH5α** **Amp^r;**
2. **E. coli BL21(DE3)** **GSTxTCSxLpHsp17(II)76.1**

Cloning: **Ligation of EcoRI/XhoI fragment of pRTLp-Hsp17(II)76.1 into EcoRI/XhoI cut pGEX 4T1**

Used for: **Expression of GSTxTCSxLp-Hsp17(II) (FGTSKHERR+ aa 1-155) in bacteria**

Constructed by/obtained from: **R. Calligaris** Date: **06/99**

Reference: **Laborbuch, Promotion** Sequencing: **RC82/RC83**



Name in text: **GST Lp-Hsp17(IIs) protein** Code N.:

Insert **Lp-Hsp17 class IIs** **RC65**

Cloning Vector: **pGEX 4T1**

Host Organisms: Functional Markers:

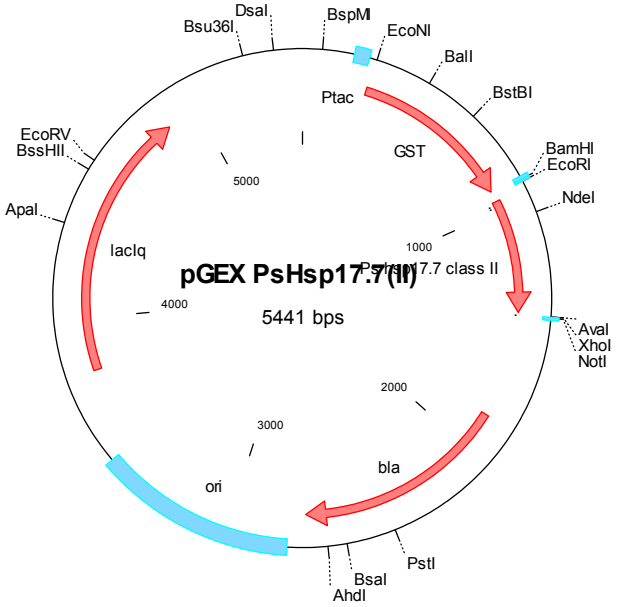
1. **E. coli** **Amp^r**
2. **E. coli BL21(DE3)** **GSTxTCSxLp-Hsp17(IIs)**

Cloning: **Ligation of EcoRI/XhoI fragment of pADHsp17(II)s into Eco/Xho cut pGEX 4T1**

Used for: **Expression of GSTxTCSxLp-Hsp17(IIs) (EFGTR+aa 7-143) in bacteria**

Constructed by/obtained from: **R. Calligaris** Date: **03/00**

Reference: **Laborbuch, Promotion** Sequencing: **RC144.2**



Name in text: **GST Ps-Hsp17.7(II) protein** Code N.:

Insert: **Ps Hsp 17.7 class II**

Cloning Vector: **pGEX 4T3**

Host Organisms: Functional Markers:

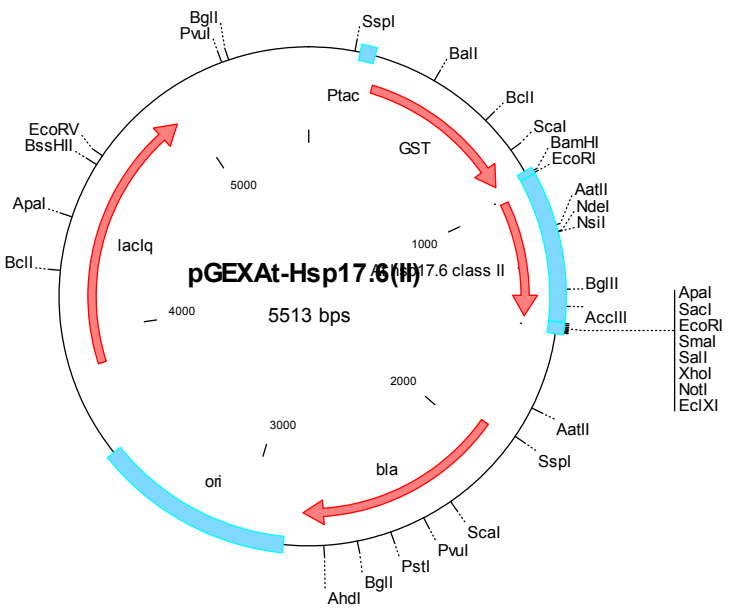
1. **E. coli** **Amp^r**
2. **E. coli BL21(DE3)** **Ps hsp 17.7-II**

Cloning: **PCR amplification of Ps hsp 17.7 in pRT101, 5´primer with EcoRI -site (Pr.474) and mutated ATG, 3´primer with XhoI site (Pr.473); insertion of EcoRI/XhoI fragment into pGEX 4T3**

Used for: **Expression of GSTxTCSxPs-Hsp 17.7-II (aa 2-157) in bacteria**

Constructed by/obtained from: **Joanna Tripp** Date: **05/99**

Reference: **Laborbuch J. Tripp Versuch 34** Sequencing: **07/99**



Name in text: **GST At-Hsp17.6(II) protein** Code N.:

Insert **At-Hsp17.6 class II** **RC 47**

Cloning Vector: **pGEX 4T3**

Host Organisms: Functional Markers:

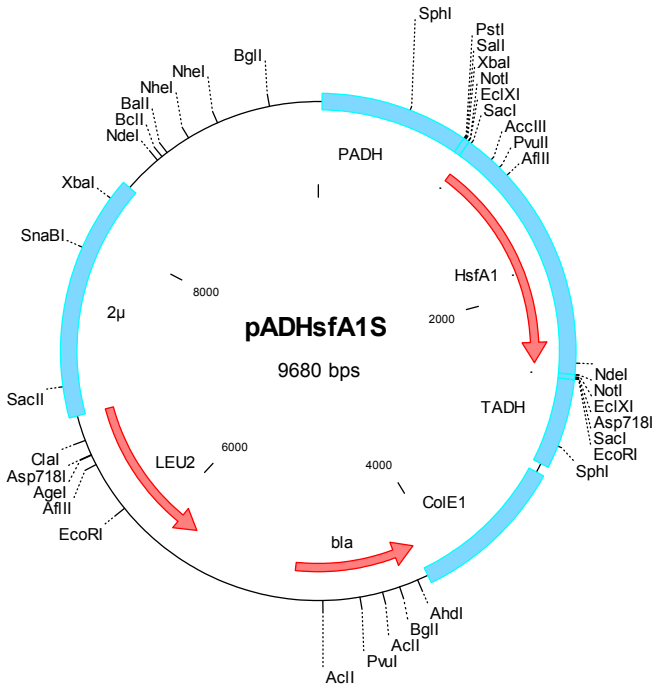
1. **E. coli** **Amp^r**
2. **E. coli BL21(DE3)** **GSTxTCSxAt-Hsp17.6(II)**

Cloning: **Ligation of EcoRI fragment of pMBI At-Hsp17.6(II) into EcoRI cut pGEX 4T3**

Used for: **Expression of GSTxTCSxAt-Hsp17.6(II) (aa1-155) in bacteria**

Constructed by/obtained from: **R. Calligaris** Date: **07/99**

Ref.: **Laborbuch** Sequencing: **RC98/RC99**



Name in text: **HsfA1** Code N.:

Insert: **Lp-HsfA1 cDNA** **Ni 12**

Cloning Vector: **pAD4Δ** (Ballester et al. Cell 15(87)5890)

Host Organisms: Functional Markers:

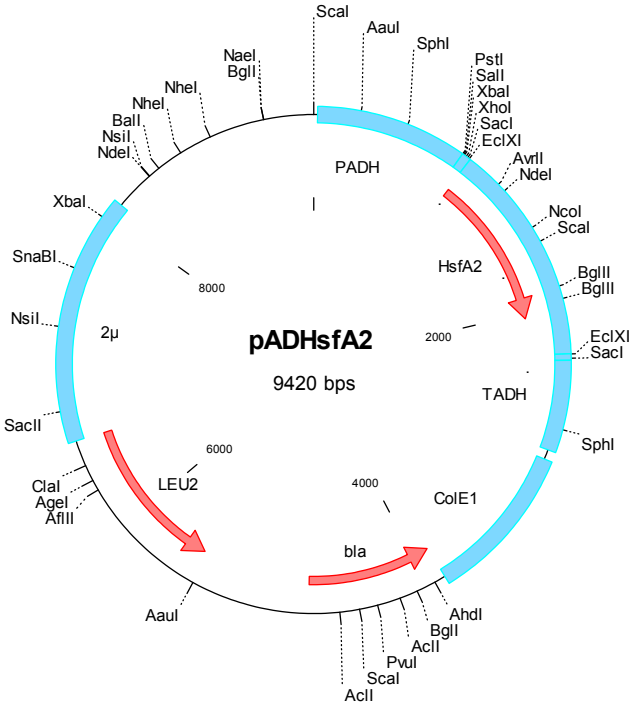
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2, HsfA1**

Cloning: **EcoRI fragment of pRTHsfA1, EcoRI filled in, subcloned into pAD4Δ/SmaI**

Used for: **Expression of Lp-HsfA1 (aa 23 to 527) in yeast cells**

Constructed by/obtained from: **E. Treuter** Date:

Ref.: **Boscheinen et al. MGG 255(97)322** Sequencing:



Name in text: **HsfA2** Code N.:

Insert: **Lp-HsfA2 cDNA** **Ni 14**

Cloning Vector: **pAD4Δ** (Ballester et al. Cell 15(87)5890)

Host Organisms: Functional Markers:

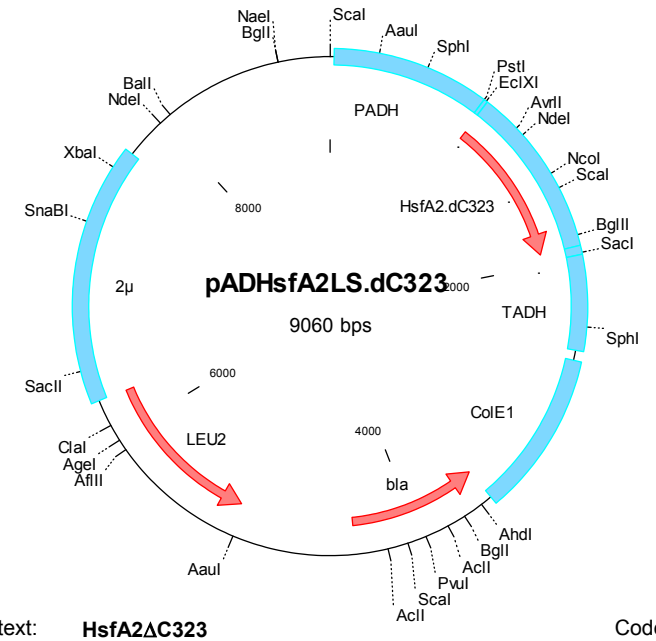
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2, HsfA2**

Cloning: **XhoI-XbaI fragment of pRTHsfA2, filled in, subcloned into pAD4Δ/SmaI**

Used for: **Expression of Lp-HsfA2 (aa 1 to 351) in yeast cells**

Constructed by/obtained from: **E. Treuter** Date:

Ref.: **Boscheinen et al. MGG 255(97)322** Sequencing:



Name in text: **HsfA2ΔC323** Code N.:

Insert: **Lp-HsfA2LS. ΔC323 cDNA** **Ni 101**

Cloning Vector: **pAD4Δ** (Ballester et al. Cell 15(87)5890)

Synonyms: **pAD30LS**

Host Organisms: Functional Markers:

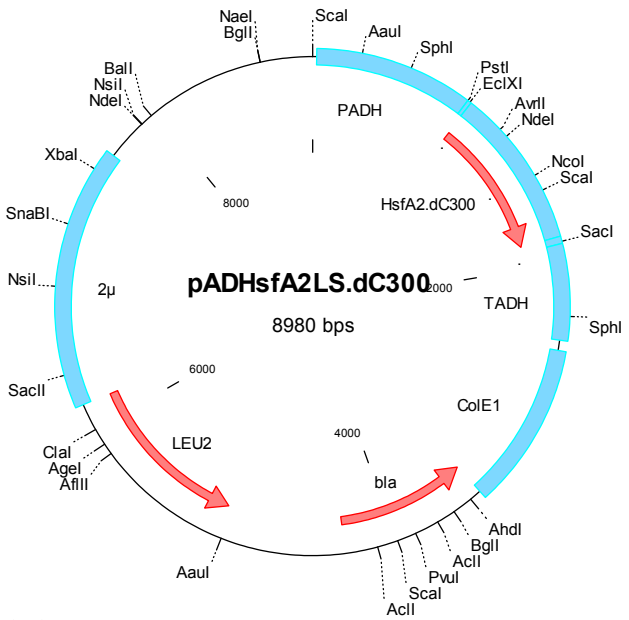
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2, HsfA2LS. ΔC323**

Cloning : **XbaI fragment of pRTHsfA2LS.dC323, filled in, Xho, subcloned into pAD4Δ, cutSal/SmaI**

Used for: **Expression of Lp-HsfA2LS. ΔC323 (aa 1 to 323+gtmhr) in yeast cells**

Constructed by/obtained from: **E. Treuter** Date:

Ref.: **Boscheinen et al. MGG 255(97)322** Sequencing:



Name in text: **HsfA2ΔC300** Code N.:

Insert: **Lp-HsfA2LS. ΔC300 cDNA** **Ni 102**

Cloning Vector: **pAD4Δ** (Ballester et al. Cell 15(87)5890)

Host Organisms: Functional Markers:

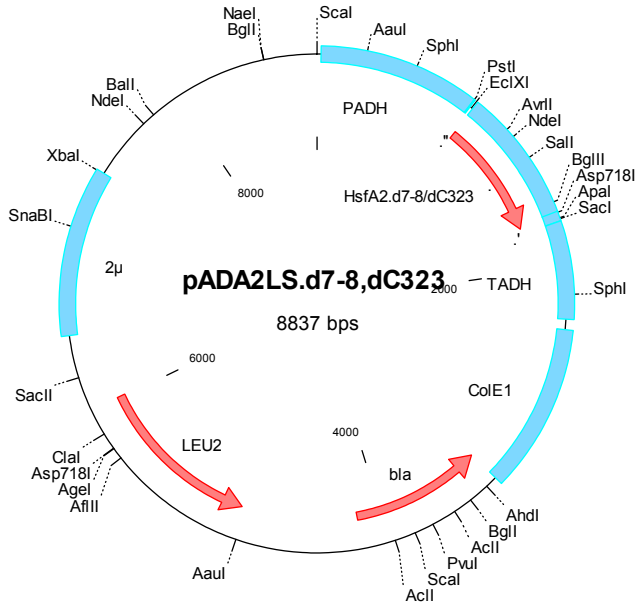
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2, HsfA2LS. ΔC300**

Cloning : **XbaI fragment of pRTHsfA2, filled in, Xho, subcloned into pAD4Δ/ cut SalI/SmaI**

Used for: **Expression of Lp-HsfA2LS. ΔC300 (aa 1 to 300+slan) in yeast cells**

Constructed by/obtained from: **E. Treuter** Date:

Ref.: **Boscheinen et al. MGG 255(97)322** Sequencing:



Name in text: **HsfA2Δ7/8, ΔC323** Code N.:

Insert: **Lp-HsfA2. Δ7/8, ΔC323 cDNA** **Ni 135**

Cloning Vector: **pAD4Δ** (Ballester et al. Cell 15(87)5890)

Synonyms: **pAD30LS. Δ7/8, ΔC323**

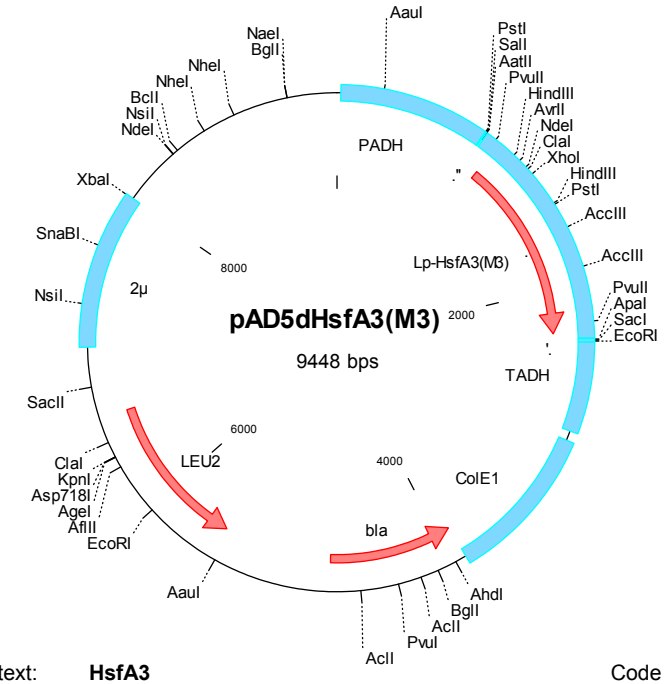
Host Organisms:	Functional Markers:
1. **E. coli**	**Amp^r**
2. **Yeast cells**	**LEU2, HsfA2. Δ7/8, ΔC323**

Cloning : **XhoI-XbaI fragment of pRTHsfA2. Δ7/8, ΔC323 subcloned into SalI/AvrII cut pAD5Δ**

Used for: **Expression of Lp-HsfA2 (aa 1-136,VD,213-323) in yeast cells**

Constructed by/obtained from: **E. Treuter** Date:

Reference: **Boscheinen et al. MGG 255(97)322** Sequencing:



Name in text: **HsfA3** Code N.:

Insert: **Lp-HsfA3(M3)** **SE 12**

Cloning Vector: **pAD5Δ**

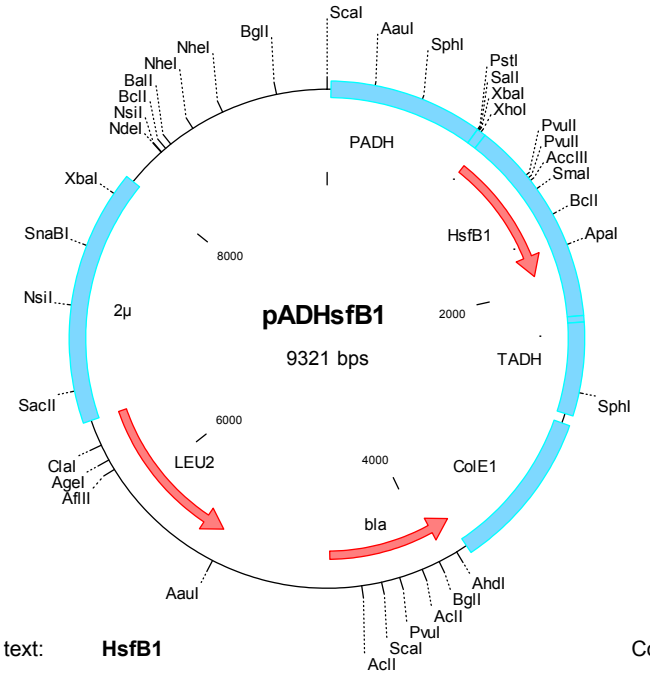
Host Organisms:	Functional Markers:
1. **E. coli**	**Amp^r**
2. **yeast**	**Leu2, HsfA3(M3)**

Cloning: **PCR amplification of A3 cDNA fragment from A3full(pBSK) using 5' primer GSP19 (SalI) and 3' primer GSP20 (XbaI); insertion of SalI/Xba fragm. into SalI/AvrII cut pAD5Δ;**

Used for: **Expression of Lp-HsfA3 (M3, aaM86-F508) in yeast cells**

Constructed by/obtained from: **Enrico Schmidt** Date:

Reference: **Bharti et al. Plant J. 22(4)1** Sequencing: Se107, SE108



Name in text: **HsfB1** Code N.:

Insert: **Lp-HsfB1 cDNA** **Ni 13**

Cloning Vector: **pAD4Δ** (Ballester et al. Cell 15(87)5890)

Host Organisms: Functional Markers:

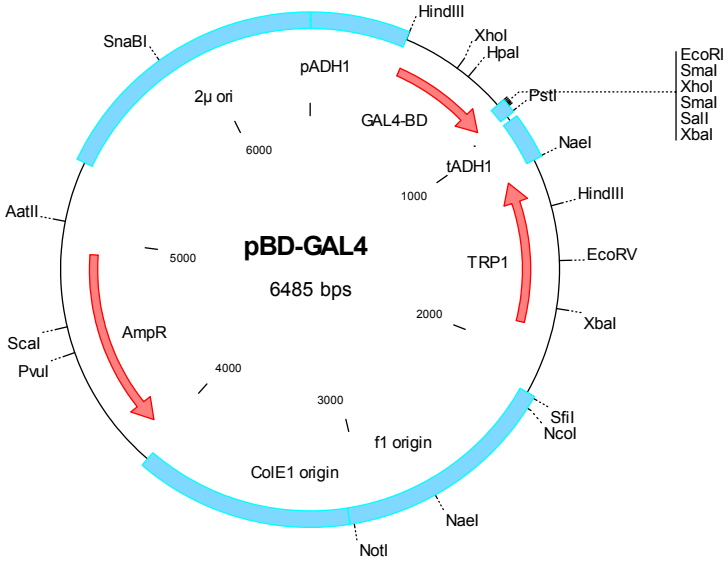
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2, HsfB1**

Cloning : **XhoI-XbaI fragment of pRTHsfB1, filled in, subcloned into pAD4Δ/SmaI**

Used for: **Expression of Lp-HsfB1 (aa 1 to 301) in yeast cells**

Constructed by/obtained from: **E. Treuter** Date:

Reference: **Boscheinen et al. MGG 255(97)322** Sequencing:



Name in text: **Gal4 BD**

Cloning Vector: **pBD-Gal4Δ** Stratagene

Host Organisms: Functional Markers:

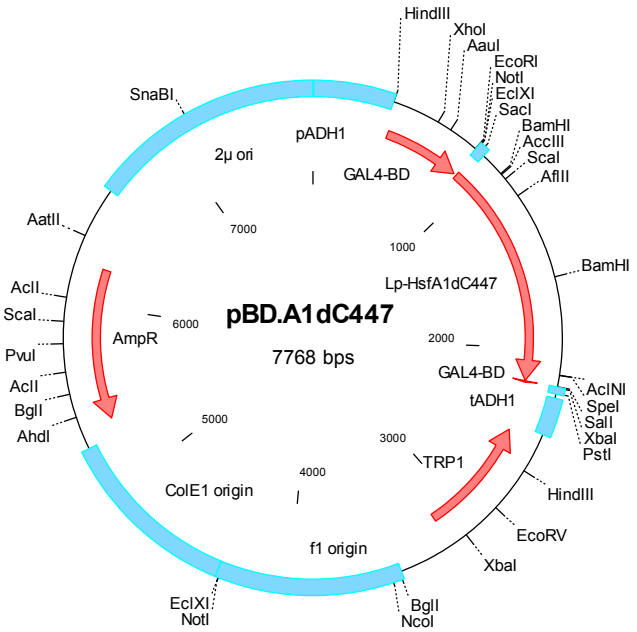
1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1**

Cloning: **Stratagene**

Used for: **High copy vector for expression of cDNA fragments fused to the Gal4 DNA binding domain (aa1-147) under control of the ADH1 promoter and terminator in yeast cells; two-hybrid bait vector**

Constructed by/obtained from: Date:

Ref.: **Stratagene Manual** Sequencing:



Name in text: **HsfA1 bait** Code N.:

Insert **Lp-HsfA1ΔC447(aa 23-447)** **RL 123**

Cloning Vector: **pBD-Gal4Δ**

Host Organisms: Functional Markers:

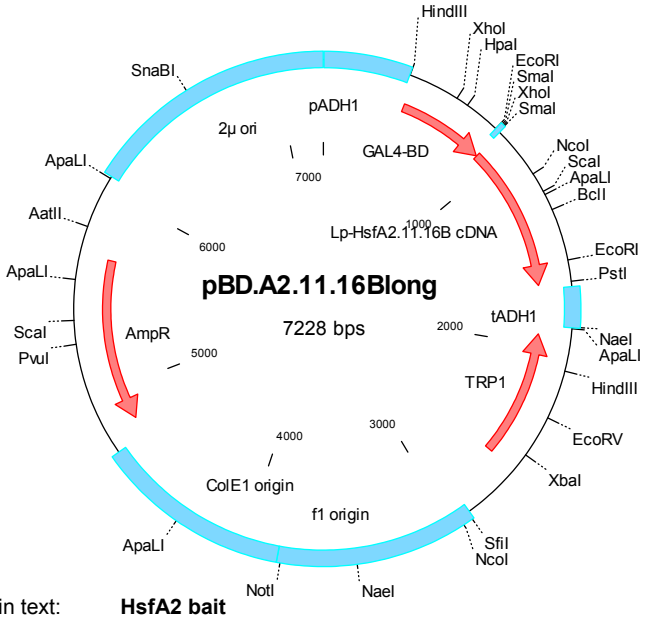
1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1; Gal4DBDxA1.dC447 fusion prot.**

Cloning: **Insertion of EcoRI/SalI fragment of pRTA1.2 into Eco/Sal cut pBDGal4 (Stratagene)**

Used for: **Expression of Gal4DBDxA1dC447 (aa23-447) in yeast, bait vector for two-hybrid screening; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R Lyck** Date:

Reference: **Promotion, Lab-Journal** Sequencing: R2H010



Name in text: **HsfA2 bait** Code N.:

Insert **Lp-HsfA2.11.16B cDNA (aa 98-351)** **RL 130**

Cloning Vector: **pBD-Gal4**

Host Organisms: Functional Markers:

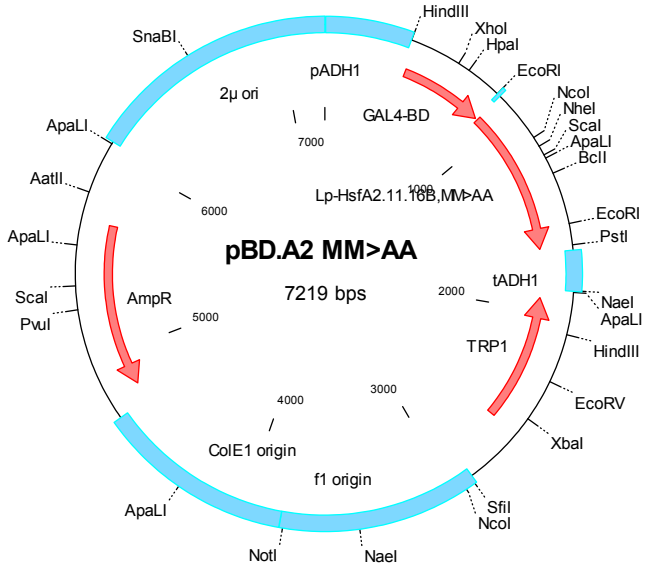
1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1; Gal4DBDxHsfA2 fusion prot.**

Cloning: **PCR amplificat. of HsfA2.11.16B (aa98-351) with 5' primer 272(XhoI) and 3' primer 273(PstI); insertion of Xho/Pst fragment into Sal/Pst cut pBDGal4;**

Used for: **Expression of Gal4-DBD(aa1-147)xHsfA2(aa98-351,W297>A,W337>E, L341>A) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Lyck** Date:

Reference: **Laborbuch, Dipl. Arb. A. Chen** Sequencing:



Name in text: **HsfA2 MM>AA bait** Code N.:

Insert: **Lp-HsfA2.11.16B MM>AA (aa 98-351; MM193/4>AA)** **RC 70**

Cloning Vector: **pBD-Gal4**

Host Organisms: Functional Markers:

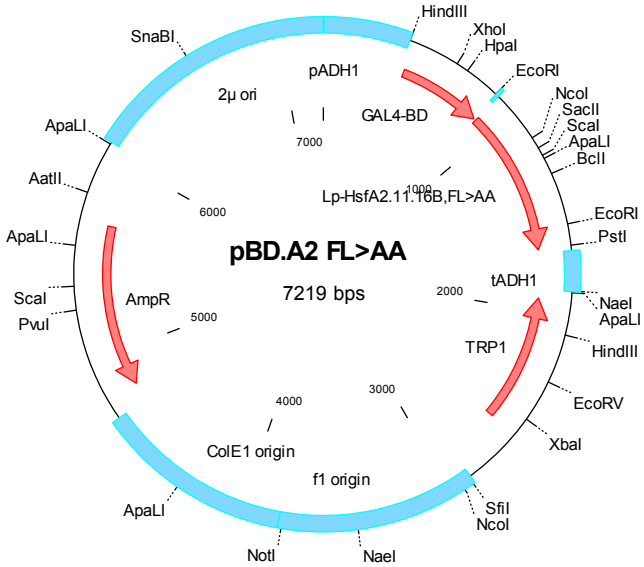
1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1; Gal4DBDxHsfA2 fusion prot.**

Cloning: **PCR amplificat. of HsfA2.11.16B (aa98-351) with 5' primer 77 and 3' primer 142; insertion of XmaI/PstI mutagenic fragment of HR-B region (MM>AA; primer 309) into XhoI/PstI cut pBDGal4; this vector: XmaI cut, Klenow fill in and ligation**

Used for: **Expression of Gal4-DBD(aa1-147)xHsfA2(aa98-351,W297>A,W337>E,L341>A; MM193/4>AA, NheI)) fusion protein in yeast**

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Ref.: **Laborbuch, Promotion** Sequencing: **RC145.2/RC150**



Name in text: **HsfA2 FL>AA bait** Code N.:

Insert: **Lp-HsfA2.11.16B FL>AA (aa 98-351; FL196/7>AA)** **RC 58**

Cloning Vector: **pBD-Gal4**

Host Organisms: Functional Markers:

1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1; Gal4DBDxHsfA2 fusion prot.**

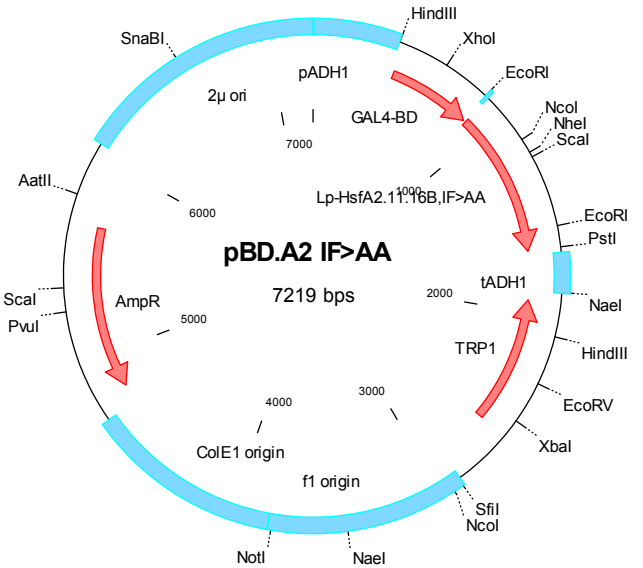
Cloning: **PCR amplificat. of HsfA2.11.16B (aa98-351) with 5' primer 77 and 3' primer 142; insertion of XmaI/PstI mutagenic fragment of HR-B region (FL>AA; primer 310) into XhoI/PstI cut pBDGal4; this vector: XmaI cut, Klenow fill in and ligation**

Used for: **Expression of Gal4-DBD(aa1-147)xHsfA2(aa98-351,W297>A,W337>**

E,L341>A; FL196/7>AA, SacII)) fusion protein in yeast; use for two-hybrid test interaction studies

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Ref.: **Laborbuch, Promotion** Sequencing: **RC127**



Name in text: **HsfA2 IF>AA bait** Code N.:

Insert: **Lp-HsfA2.11.16B IF>AA (aa 98-351; IF200/1>AA)** **RC 59**

Cloning Vector: **pBD-Gal4**

Host Organisms: Functional Markers:

1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1; Gal4DBDxHsfA2 fusion prot.**

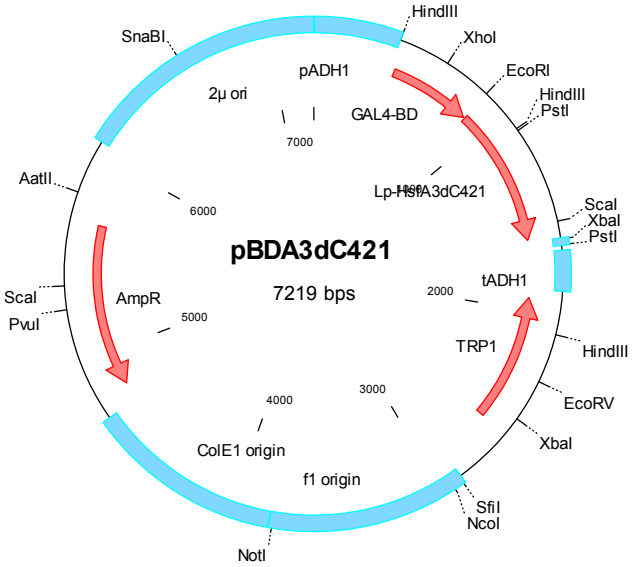
Cloning: **PCR amplificat. of HsfA2.11.16B (aa98-351) with 5' primer 77 and 3' primer 142; insertion of XmaI/PstI mutagenic fragment of HR-B region (IF>AA; primer 311) into XhoI/PstI cut pBDGal4; this vector: XmaI cut, Klenow fill in and ligation**

Used for: **Expression of Gal4-DBD(aa1-147)xHsfA2(aa98-351,W297>A,W337>**

E,L341>A; IF200/1>AA, NheI)) fusion protein in yeast; use for two-hybrid test interaction studies

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Ref.: **Laborbuch, Promotion** Sequencing: **RC128**



Name in text: **HsfA3 bait** Code N.:

Insert **Lp-HsfA3dC421 (aa 177-421)** **KB 30**

Cloning Vector: **pBD-Gal4**

Host Organisms: Functional Markers:

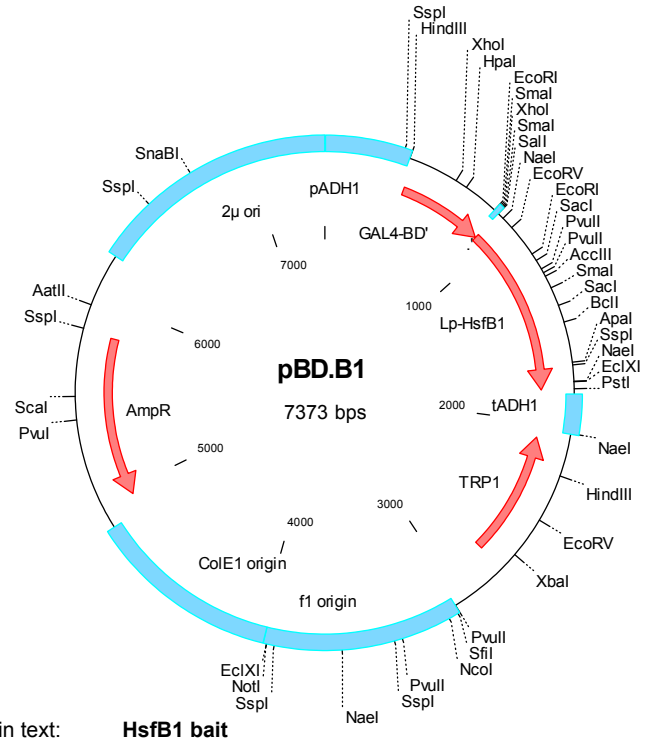
1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1, Gal4DBDxHsfA3(aa177-421)**

Cloning: **PCR amplification of N-terminal region of HsfA3 (bp 751-1624 from pBDA3- 47.1) with 5' primer 77 and 3' primer 506; ligation of EcoR I/ Xho I fragment into EcoR I/Sal I cut pBDGal4**

Used for: **Expression of Gal4-DBD(aa1-147)xHsfA3 (aa177-421) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **K. Bharti** Date: **1999**

Ref.: **Laborbuch** Sequencing:



Name in text: **HsfB1 bait** Code N.:

Insert **Lp-HsfB1 (aa 1-301)** **RL 125**

Cloning Vector: **pBD-Gal4Δ**

Host Organisms: Functional Markers:

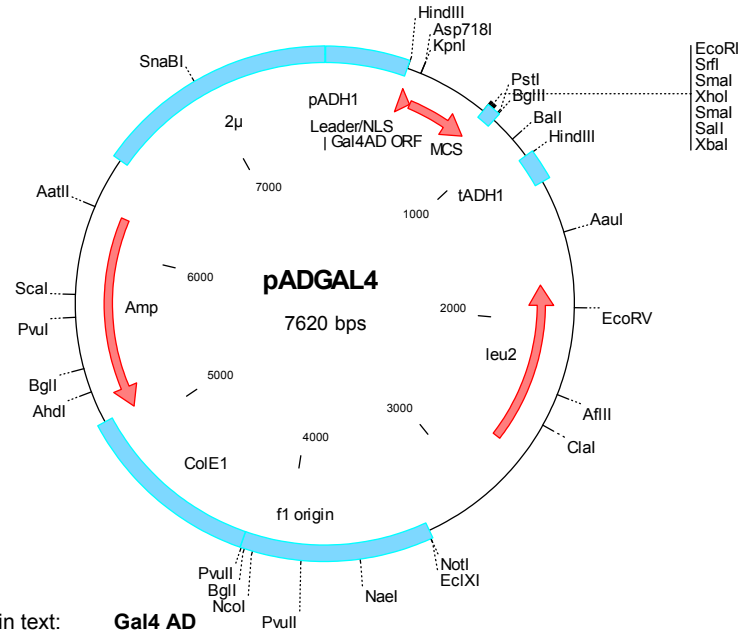
1. **E. coli** **Amp^r**
2. **Yeast cells** **TRP1; Gal4DBDxHsfB1 fusion prot.**

Cloning: **Use of Sal/Pst cut pBDGal4 (Stratagene for insertion of SalI/PstI fragment from PCR-amplified fragment of pJC2437S using primers 138 (5' Sal site) and 139 (3' Pst site**

Used for: **Expression of Gal4DBDxHsfB1 fusion protein in yeast, bait for two-hybrid screening; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R Lyck** Date:

Reference: **Promotion, Lab-Journal** Sequencing: R2H010



Name in text: **Gal4 AD**

Cloning Vector: **pAD-Gal4** Stratagene

Host Organisms: Functional Markers:

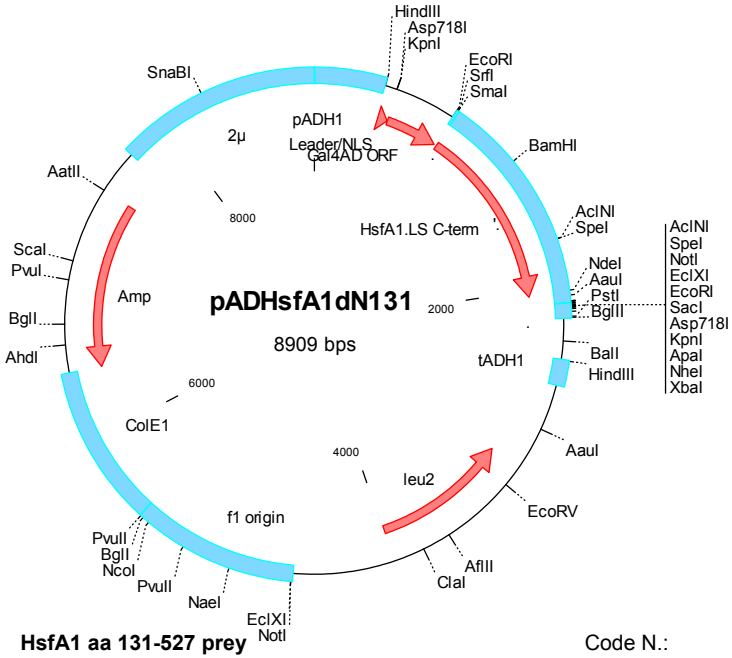
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2**

Cloning: **From Stratagene**

Used for: **High copy vector for expression of cDNA fragments fused to Gal4 AD (aa 761-881) under control of the ADH1 promoter and terminator in yeast cells; two-hybrid screening: prey vector**

Constructed by/obtained from: Date:

Ref.: **Stratagene Manual** Sequencing:



Name in text: **HsfA1 aa 131-527 prey** Code N.:

Insert: **Lp-HsfA1 C-term.** **SE 1**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

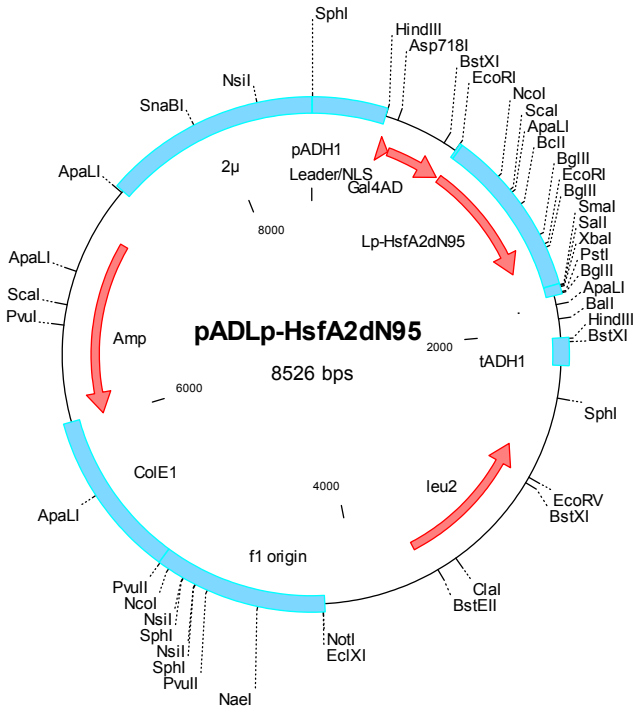
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA1 C-term**

Cloning: **Ligation of Sal/Xba fragment from pRTA1.M2(pRL111) into Xho/Xba cut pADGal4**

Used for: **Expression of Gal4ADxHsfA1(aa 131-527) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **E. Schmidt** Date:

Reference: **Diplomarbeit** Sequencing:



Name in text: **HsfA2 aa 95-351 prey** Code N.:

Insert: **Lp-HsfA2 C-term.** **pAD39.1; RL39**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

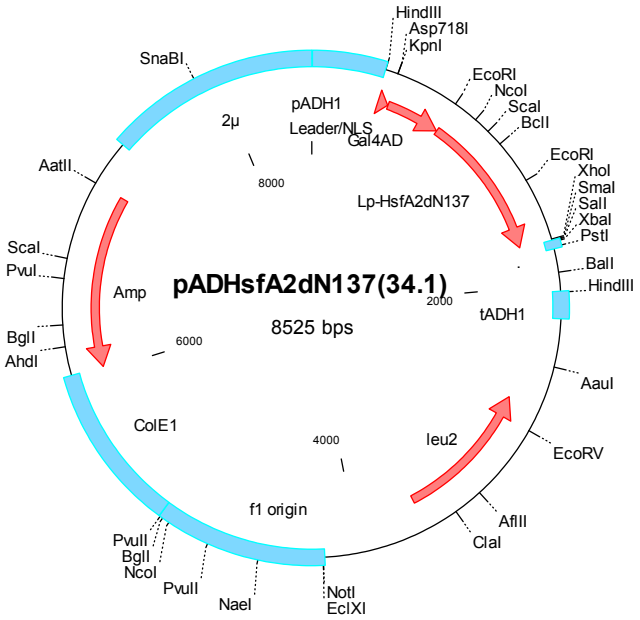
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 C-term**

Cloning **C-terminal fragment of Lp-HsfA2 (aa 95-351) fused to Gal4AD; isolated as interaction partner of Lp-HsfA1 in a two-hybrid screening;**

Used for: **Expression of Gal4ADxHsfA2(aa 95-351) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Lyck** Date:

Ref.: **Promotion** Sequencing:



Name in text: **HsfA2 aa 137-351 prey** Code N.:

Insert: **Lp-HsfA2 C-term. (aa137-351+3'UTR)** **pAD34.1; RL34**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

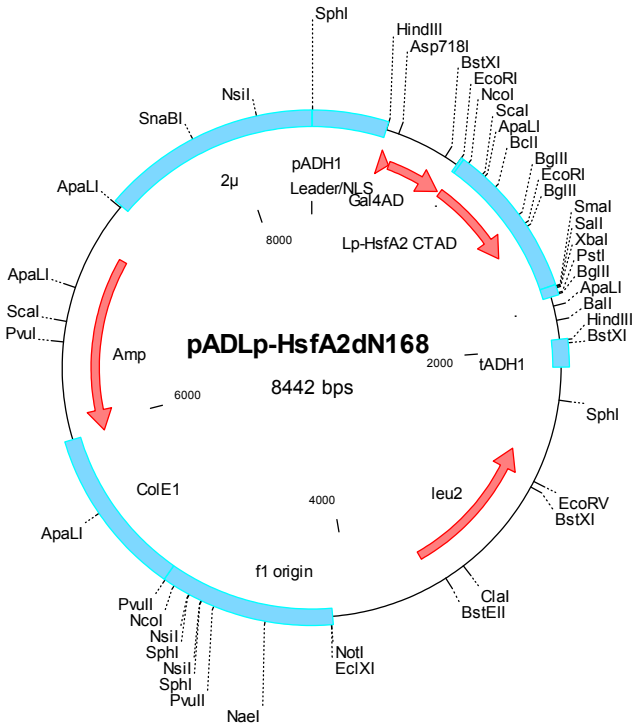
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 (aa137-351)**

Cloning: **C-terminal fragment of Lp-HsfA2 (aa 137-351) fused to Gal4AD; isolated as interaction partner of Lp-HsfA1 (RL123) in a two-hybrid screening**

Used for: **Expression of Gal4ADxHsfA2(aa 137-351) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Lyck** Date:

Ref.: **Promotion** Sequencing: SE 721, 722



Name in text: **HsfA2 aa 168-351 prey** Code N.:

Insert: **Lp-HsfA2 C-term.** pAD145.1; RL145

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

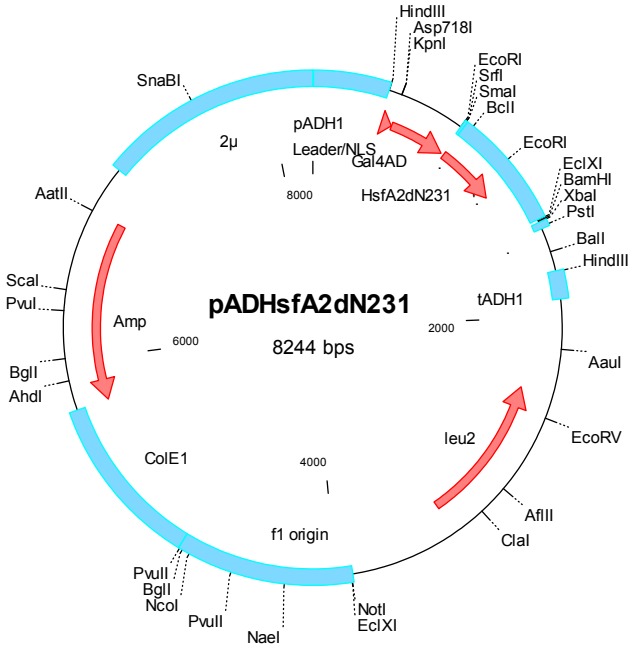
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 C-term**

Cloning: **C-terminal fragment of Lp-HsfA2 (aa168-351) fused to Gal4AD; isolated as interaction partner of Lp-HsfA1 in a two-hybrid screening;**

Used for: **Expression of Gal4ADxHsfA2(aa168-351) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Lyck** Date:

Ref.: **Promotion** Sequencing:



Name in text: **HsfA2 aa 231-351 prey** Code N:

Insert: **Lp-HsfA2 C-term.** **SE2**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

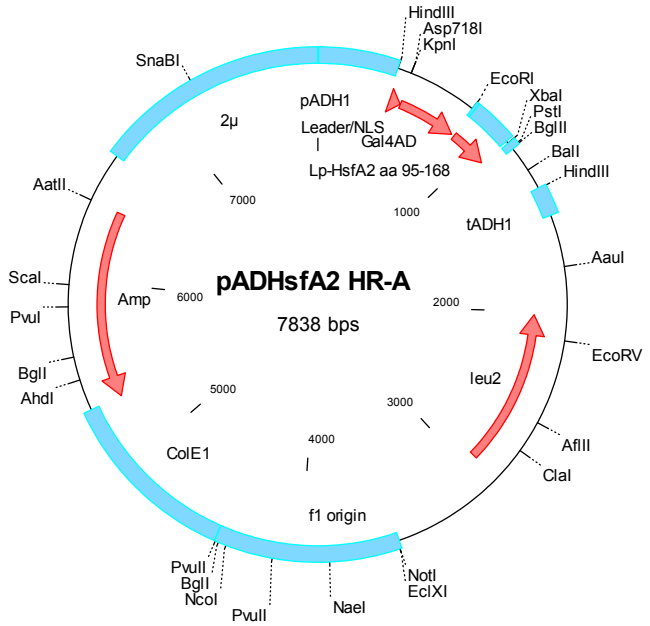
1. **E. coli** **Amp^r**
2. **yeast** **Leu2 Gal4ADxLp-HsfA2 C-term**

Cloning: **C-terminal fragment of Lp-HsfA2 (aa231-351) fused to Gal4AD; ligation of SalI/XbaI from pRTA2.M3(pRL108) into pADGAL4 cut XhoI/XbaI**

Used for: **Expression of Gal4ADxHsfA2(aa231-351) in yeast cells use for two-hybrid test interaction studies**

Constructed by/obtained from: **E. Schmidt** Date: May 1997

Reference: **Diplomarbeit** Sequencing: **E. Schmidt**



Name in text: **HsfA2 aa 95-168 prey** Code N:

Insert: **Lp-HsfA2 N-term.** **RC66**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

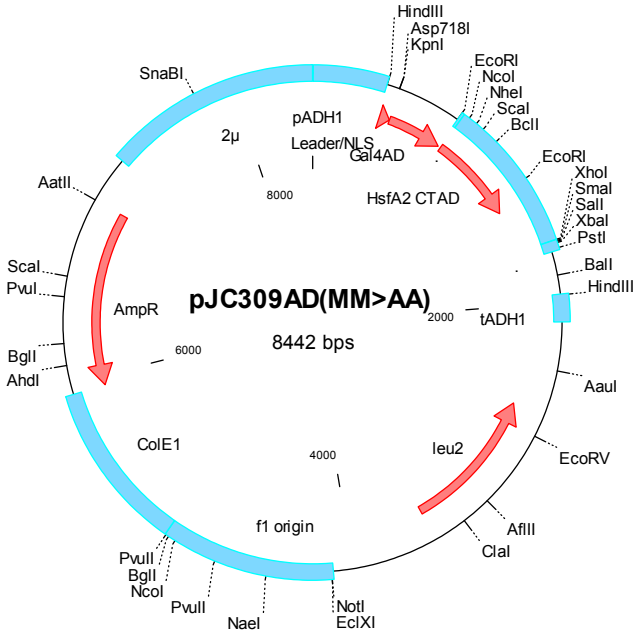
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 N-term**

Cloning: **PCR amplification of N-terminal region of HsfA2 (template: HsfA2dN95-351; RL39) with 5' primer 78 and 3' primer 512; ligation of EcoR I/XbaI fragment into EcoRI/XbaI cut pADGal4**

Used for: **Expression of Gal4ADxHsfA2(aa 95-168, +8aa) in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC146**



Name in text: **HsfA2 aa 168-351, MM>AA prey** Code N:

Insert: **Lp-HsfA2 CTAD (aa168-351, MM/AA)** **pJC309**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

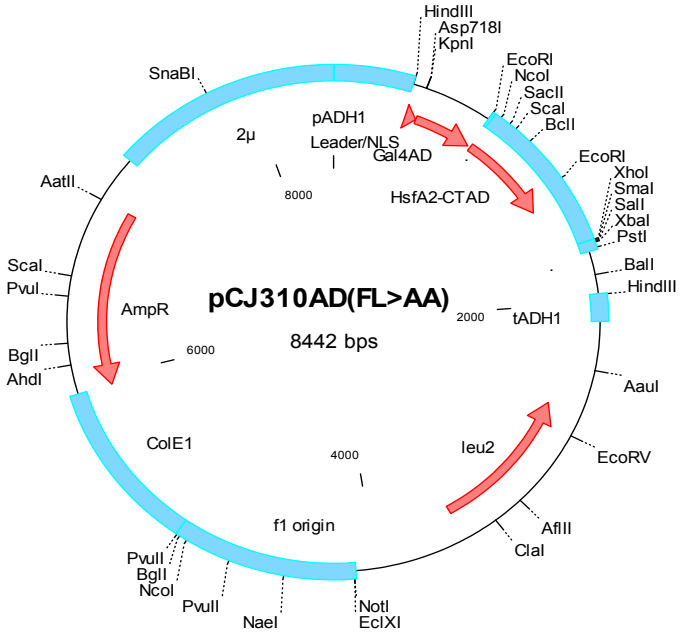
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 CTAD**

Cloning: **C-terminal fragment of Lp-HsfA2 (aa168-351) fused to Gal4AD; isolated as interaction partner of Lp-HsfA1 in a two-hybrid screening; PCR mutagenesis of MM>AA in HR-B region (NheI);**

Used for: **Expression of Gal4ADXLp-HsfA2(aa168-351; MM193/4>AA,NheI) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **C. Janista** Date: **1999**

Reference: **Diplomarbeit** Sequencing:



Name in text: **HsfA2 aa 168-351, FL>AA prey** Code N:

Insert: **Lp-HsfA2 CTAD (aa168-351, FL>AA)** **pJC310**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

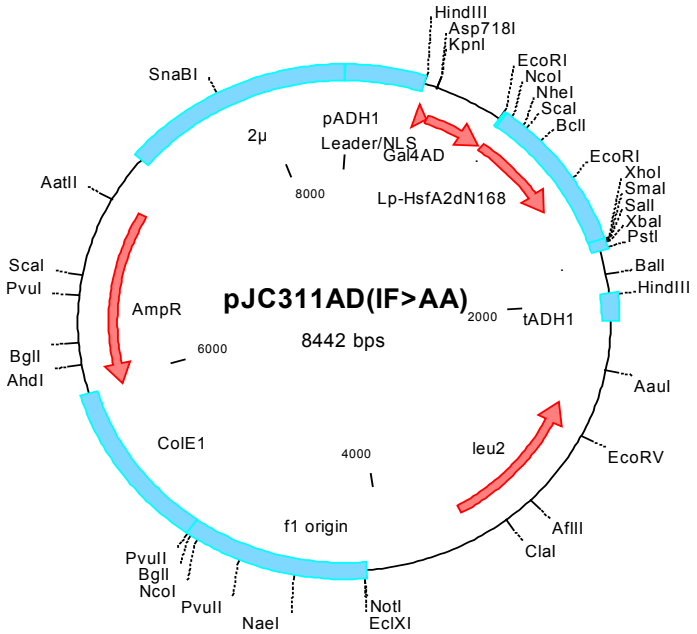
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 CTAD**

Cloning: **C-terminal fragment of Lp-HsfA2 (aa168-351) fused to Gal4AD; isolated as interaction partner of Lp-HsfA1 in a two-hybrid screening; PCR mutagenesis of FL>AA in HR-B region (SacII);**

Used for: **Expression of Gal4ADXLp-HsfA2(aa168-351; FL196/7>AA,SacII) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **C. Janista** Date:

Reference: **Diplomarbeit** Sequencing:



Name in text: **HsfA2 aa 168-351, IF>AA prey** Code N:

Insert: **Lp-HsfA2 CTAD (aa168-351, IF>AA)** **pJC311**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

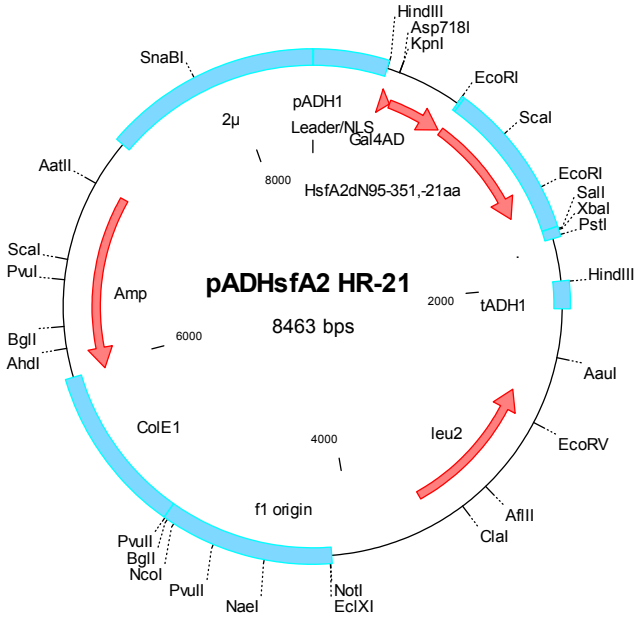
1. **E. coli** **Amp^r**
2. **yeast** **Leu2, Gal4ADxLp-HsfA2 CTAD**

Cloning: **C-terminal fragment of Lp-HsfA2 (aa168-351) fused to Gal4AD; isolated as interaction partner of Lp-HsfA1 in a two-hybrid screening; PCR mutagenesis of IF>AA in HR-B region (NheI);**

Used for: **Expression of Gal4ADXLp-HsfA2(aa168-351; IF200/1>AA,NheI) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **C. Janista** Date: **1999**

Reference: **Diplomarbeit** Sequencing:



Name in text: **HsfA2 aa 95-351, HR-21 prey** Code N.:

Insert: **Lp-HsfA2 dN95-351,-21aa** **RC67**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

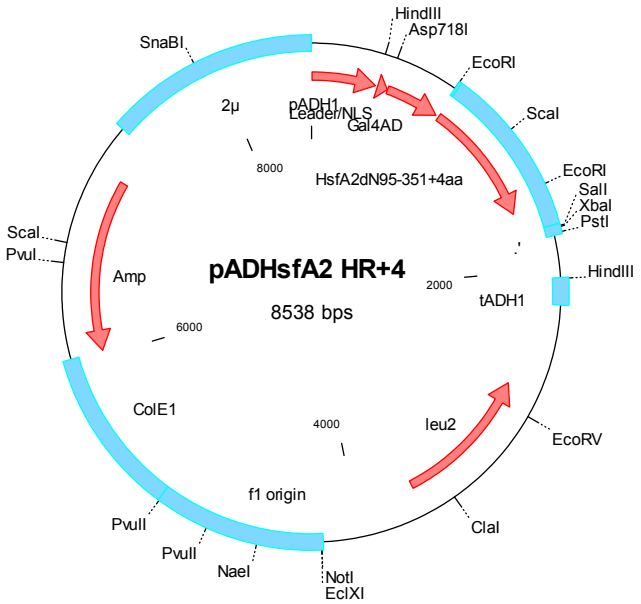
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 C-term**

Cloning: **PCR amplification of C-terminal region of HsfA2 (template: HsfA2 dN95-351; RL39) with 5' primer 513 (mutation HR-21) and 3' primer 141; ligation of BssH II/XbaI fragment into BssH II/XbaI cut pADHsfA2 HR-A (RC66)**

Used for: **Expression of Gal4ADxHsfA2(aa95-168,+AR, aa192-351) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC147**



Name in text: **HsfA2 aa 95-351, HR+4 prey** Code N.:

Insert: **Lp-HsfA2 dN95-351,+4aa** **RC68**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

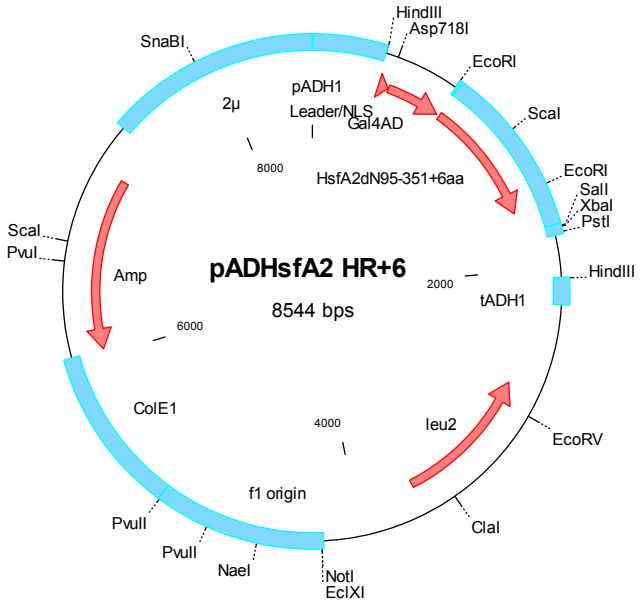
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 C-term**

Cloning: **PCR amplification of C-terminal region of HsfA2 (template: HsfA2 dN95-351; RL39) with 5' primer 514 (mutation HR+4) and 3' primer 141; ligation of BssH II/XbaI fragment into BssH II/XbaI cut pADHsfA2 HR-A (RC66)**

Used for: **Expression of Gal4ADxHsfA2(aa95-168,+ARGA, aa169-351) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Ref.: **Laborbuch, Promotion** Sequencing: **RC148**



Name in text: **HsfA2 aa 95-351, HR+6 prey** Code N.:

Insert: **Lp-HsfA2 dN95-351,+6aa** **RC69**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

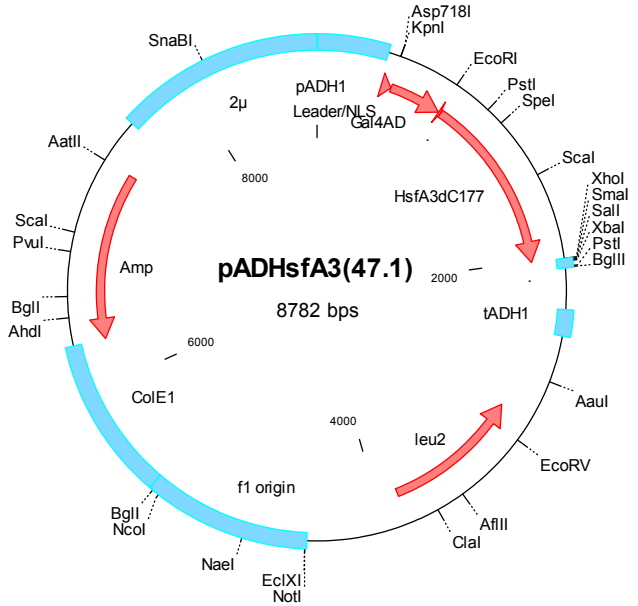
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA2 C-term**

Cloning: **PCR amplification of C-terminal region of HsfA2 (template: HsfA2 dN95-351; RL39) with 5' primer 515 (mutation HR+6) and 3' primer 141; ligation of BssH II/XbaI fragment into BssH II/XbaI cut pADHsfA2 HR-A (RC66)**

Used for: **Expression of Gal4ADxHsfA2(aa95-168,+ARGVGA, aa169-351) in yeast cells; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Ref.: **Laborbuch, Promotion** Sequencing: **RC149**



Name in text: **HsfA3 aa 177-508 prey** Code N.:

Insert: **Lp-HsfA3 C-term.** **pAD47.1; RL47**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

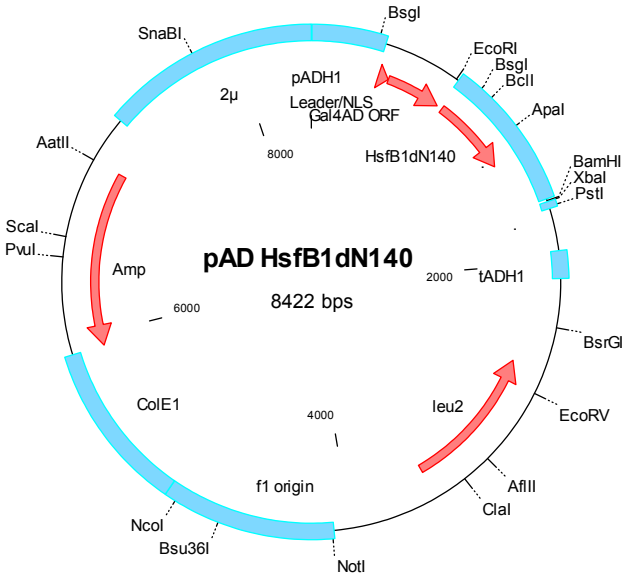
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxLp-HsfA3**

Cloning: **Stratagene prey vector with C-terminal HsfA3 fragment (aa177-508+3'UTL), fished with HsfA1(aa23-447) bait**

Used for: **Expression of Gal4ADXLp-HsfA3(aa177-508) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **Ruth Lyck** Date:

Reference: **Promotion** Sequencing:



Name in text: **HsfB1aa 140-301 prey** Code N.:

Insert **Lp-HsfB1 C-ter (aa 140-301)** **RC71**

Cloning Vector: **pAD-Gal4Δ**

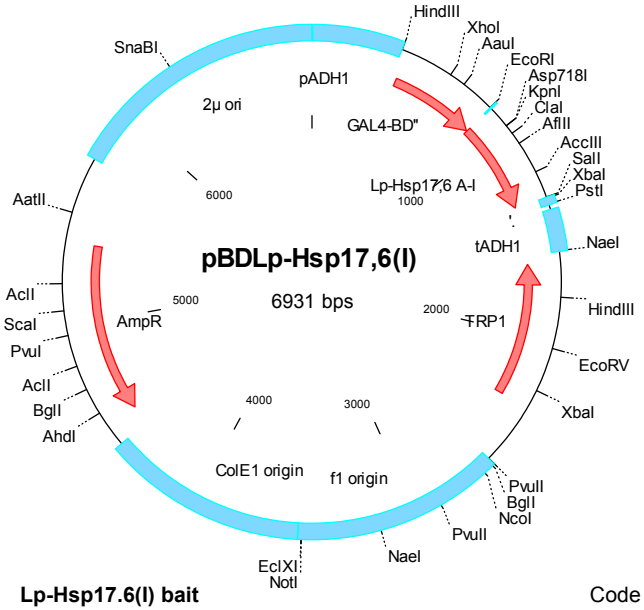
Host Organisms:	Functional Markers:
1. **E. coli**	**Amp^r**
2. **Yeast cells**	**TRP1; Gal4DBDxHsfB1 fusion prot.**

Cloning: **C-terminal fragment of Lp-HsfB1(aa140-301) fused to Gal4AD; ligation of EcoRI/XbaI from pRLHsfB1SdN139 into pADGal4 cut EcoRI/XbaI**

Used for: **Expression of Gal4ADxHsfB1(aa 140-301) fusion protein in yeast; use for two-hybrid test interaction studies**

Constructed by/obtained from: **R. Calligaris** Date: **03/2000**

Reference: LABORBUCH, PROMOTION Sequencing: **RC151/RC152**



Name in text: **Lp-Hsp17.6(I) bait** Code N.:

Insert: **Lp-Hsp 17,6 class I** **MK 49**

Cloning Vector: **pBD-GAL4**

Host Organisms: Functional Markers:

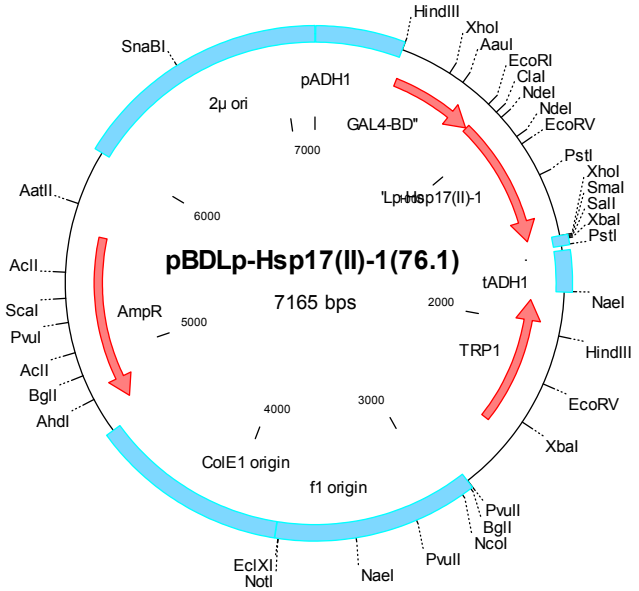
1. **E. coli** **Amp^r**
2. **Yeast** **Trp1; Gal4DBDxLpHsp17,6(I)**

Cloning strategy: **PCR amplification of LpHsp17,6(I) from corresponding pRT vector with Pr.409F(EcoRI) and Pr.410R(SalI); insertion of Eco/Sal fragment into pBDGal4;**

Used for: **Expression of GalDBD (aa 1-147) xLp-Hsp17.6(I) (aa 1-154) in yeast, TH interaction**

Constructed by/obtained from: **Marc Kirschner** Date: **10/99**

Reference: Sequencing:



Name in text: **Lp-Hsp17(II) bait** Code N.:

Insert: **Lp-Hsp17 class II, type 1** **MK 50**

Cloning Vector: **pBD-GAL4**

Host Organisms: Functional Markers:

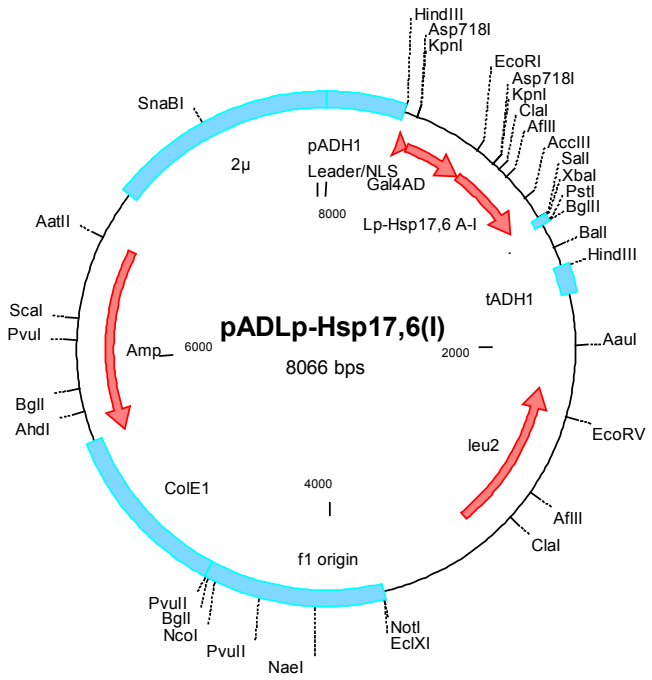
1. **E. coli** **Amp^r**
2. **Yeast** **Trp1; Gal4DBDxLp-Hsp17 (II)**

Cloning : **Ligation of EcoRI/SalI fragment from pAD clone 76.1 into pBDGal4;**

Used for: **Expression of pBDGal4xLp-Hsp17(II)-1 (aa 1-155) in yeast; T-H assays**

Constructed by/obtained from: **Marc Kirschner** Date: **06/99**

Ref.: **Laborbuch MK 182F** Sequencing:



Name in text: **Lp-Hsp17.6(I) prey** Code N.:

Insert: **Lp-Hsp 17,6 class I** **MK 48**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

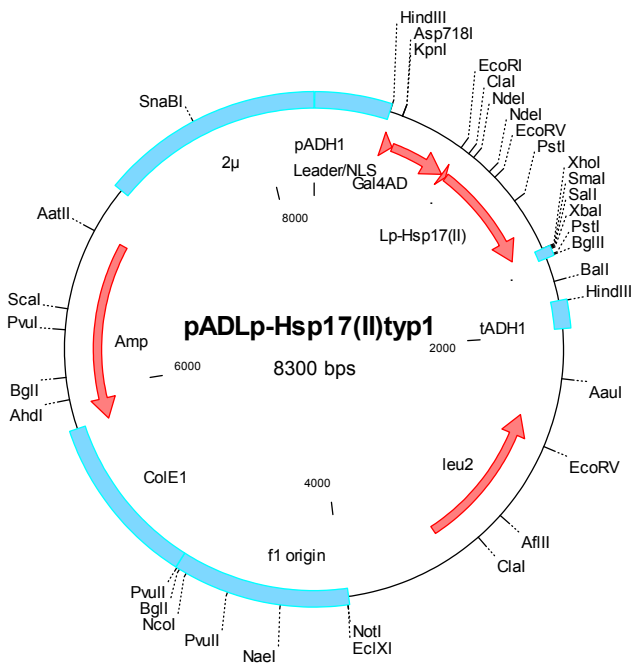
1. **E. coli** **Amp^r**
2. **Yeast** **Leu2; Gal4ADxLpHsp17,6(I)**

Cloning strategy: **PCR amplification of LpHsp17,6(I) from corresponding pRT vector with Pr.409F(EcoRI) and Pr.410R(SalI); insertion of Eco/Sal fragment into pADGal4;**

Used for: **Expression of GalDBD (aa 761-881) xLp-Hsp17.6(I) (aa 1-154) in yeast, TH interaction**

Constructed by/obtained from: **Marc Kirschner** Date: **10/99**

Reference: Sequencing:



Name in text: **Lp-Hsp17(II) prey** Code N.:

Cloning Vector: **pADLp-Hsp17(II) type 1** **AC clone 76.1**

Host Organisms: Functional Markers:

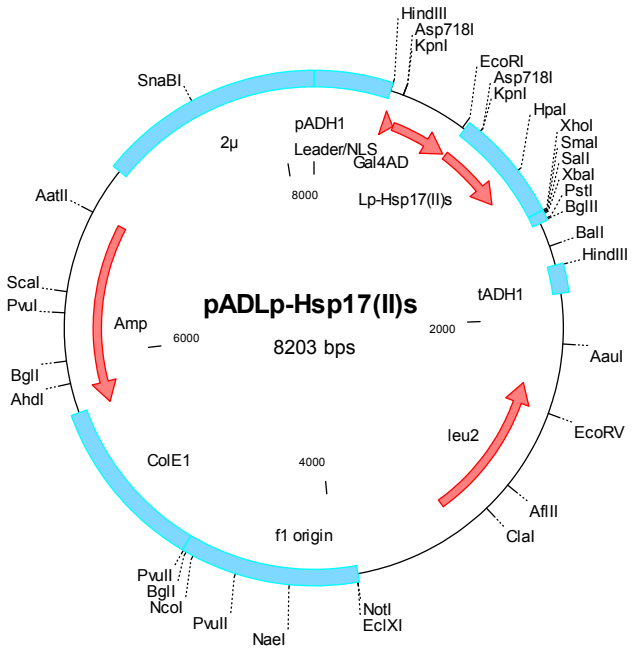
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2; Lp-Hsp17(II)typ1**

Cloning: **Isolated by two hybrid screening with HsfA2 bait;**

Used for: **Expression of pADGal4xLp-Hsp17(II) (IEFGTSKHERR+aa1-155+150 nucl UTR (Type 1))**

Constructed by/obtained from: A. Chen Date:

Reference: **Dipl. Thesis** Sequencing:



Name in text: **Lp-Hsp17(II)s prey** Code N.:

Cloning Vector: **pADLp-Hsp17(II)s** **MP clone I.8.2**

Host Organisms: Functional Markers:

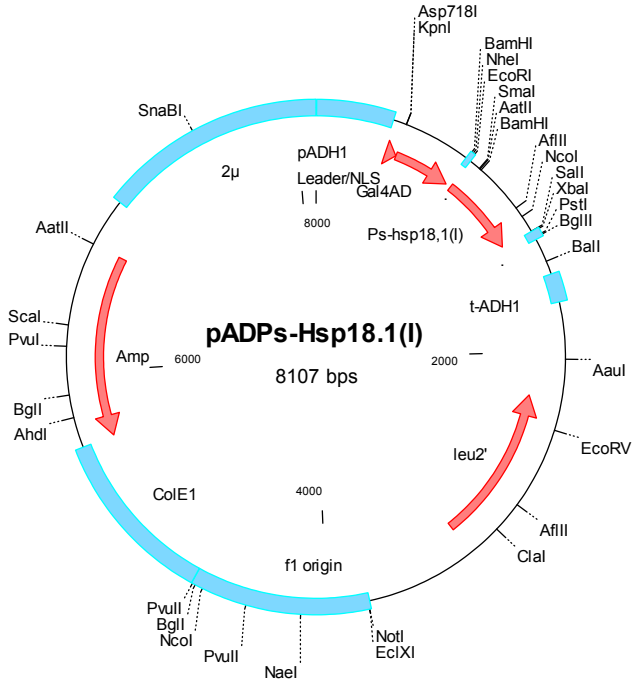
1. **E. coli** **Amp^r**
2. **Yeast cells** **LEU2, Lp-Hsp17(II)s**

Cloning: **Isolated by two hybrid screening with Hsp17(I) bait;**

Used for: **Expression of pADGal4xLp-Hsp17(II) (IEFGTR+ aa 7-143+150 nucl UTR)**

Constructed by/obtained from: **M. Port** Date:

Ref.: **Laborbuch** Sequencing:



Name in text: **Ps-Hsp18.1(I) prey** Code N.:

Insert: **Ps-Hsp 18,1 class I** **MK 40**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

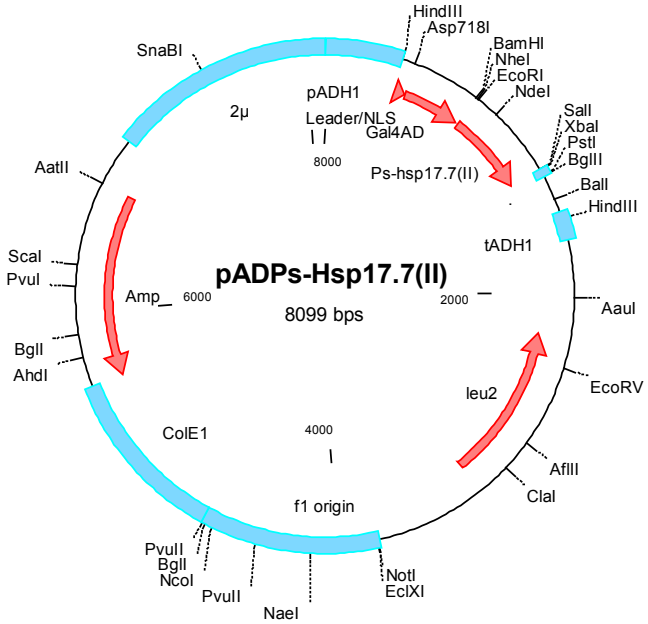
1. **E. coli** **Amp^r**
2. **yeast** **Leu2; Gal4ADxHsp 18,1 class I**

Cloning: **PCR amplification of Hsp18.1 cDNA from pRTPs-Hsp18.1(I) with 5' primer(295, EcoRI) and 3' primer (296,SalI); insertion into EcoRI/SalI cut pADGal4 (Stratagene);**

Used for: **Expression of Gal4AD(aa761-881)xHsp18.1(aa 1-158) fusion protein in yeast; T-H- interaction**

Constructed by/obtained from: **Marc Kirschner** Date: **10/98**

Ref.: Sequencing:



Name in text: **Ps-Hsp17.7(II) prey** Code N.:

Insert: **Ps-Hsp 17,7 class II** **MK45**

Cloning Vector: **pAD-GAL4**

Host Organisms: Functional Markers:

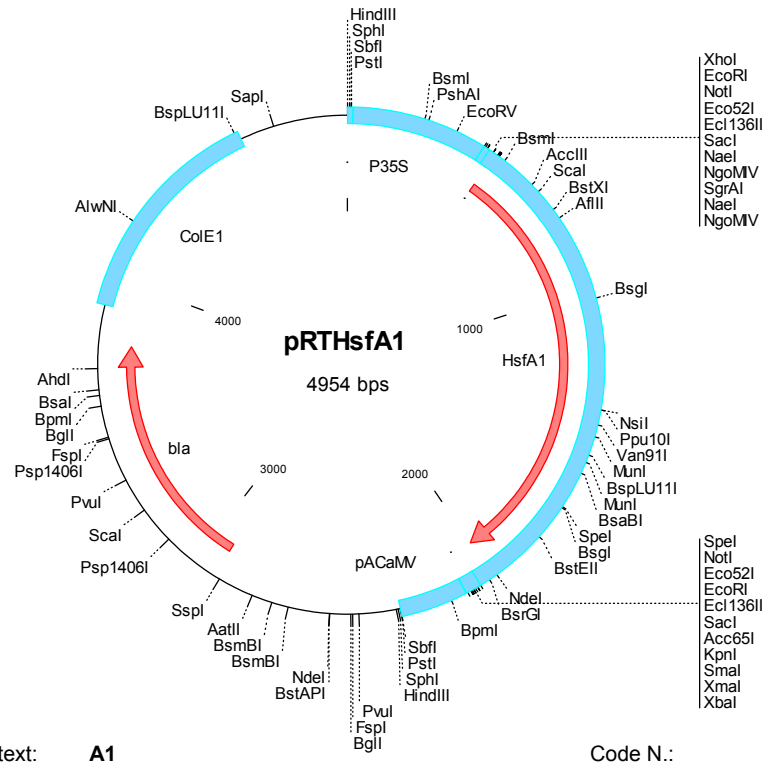
1. **E. coli** **Amp^r**
2. **yeast** **Leu2 Hsp 17,7 class II**

Cloning: **PCR amplification of Hsp17.7 cDNA from pRTPs-Hsp17.7(II) with 5' primer (EcoRI) and 3' primer (SalI); insertion into EcoRI/SalI cut pADGal4 (Stratagene);**

Used for: **Expression of Gal4ADXHsp17.7 (aa 1-157) fusion protein in yeast; T-H-interaction**

Constructed by/obtained from: **Marc Kirschner** Date: **10/98**

Reference: Sequencing:



Name in text: **A1** Code N.:

Insert: **Lp-HsfA1-cDNA** **ET 5, 7, 14**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Synonyms: **EHSF1.8S, pRTHsf8S**

Host Organisms: Functional Markers:

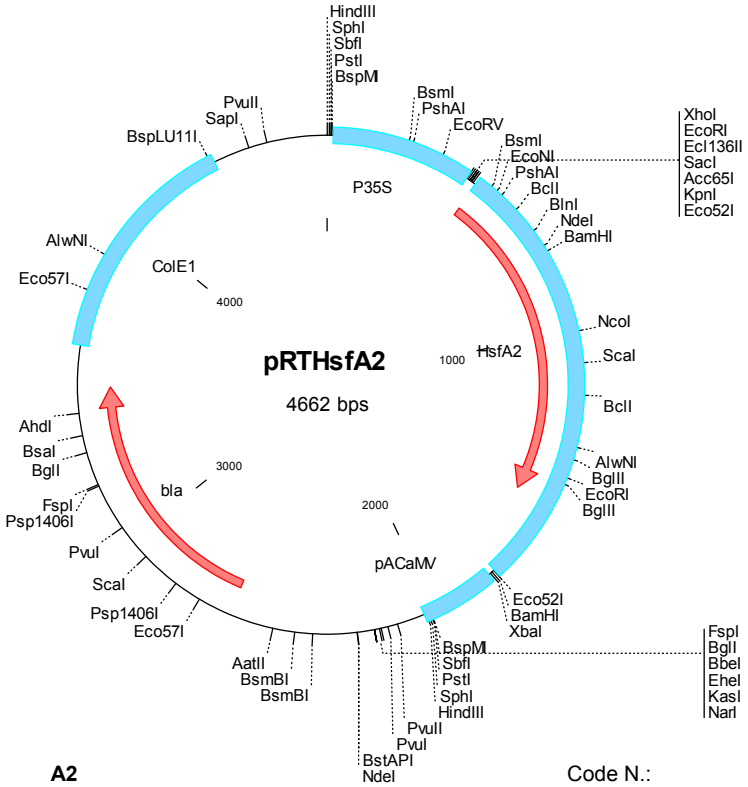
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA1**

Cloning Strategy: **EcoRI fragment of pHSFT8/EcoRI cloned into pRT101/EcoRI**

Used for: **Expression of HsfA1 (aa 23 to 527) in plant cells**

Constructed by/obtained from: **E. Treuter** Date:

Ref.: **Treuter et al. MGG 240(93)113** Sequencing:



Name in text: **A2** Code N.:

Insert: **Lp-HsfA2 cDNA** **ET 345, 391, 875**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Synonyms: **EHSF1.35S, pRT35S**

Host Organisms: Functional Markers:

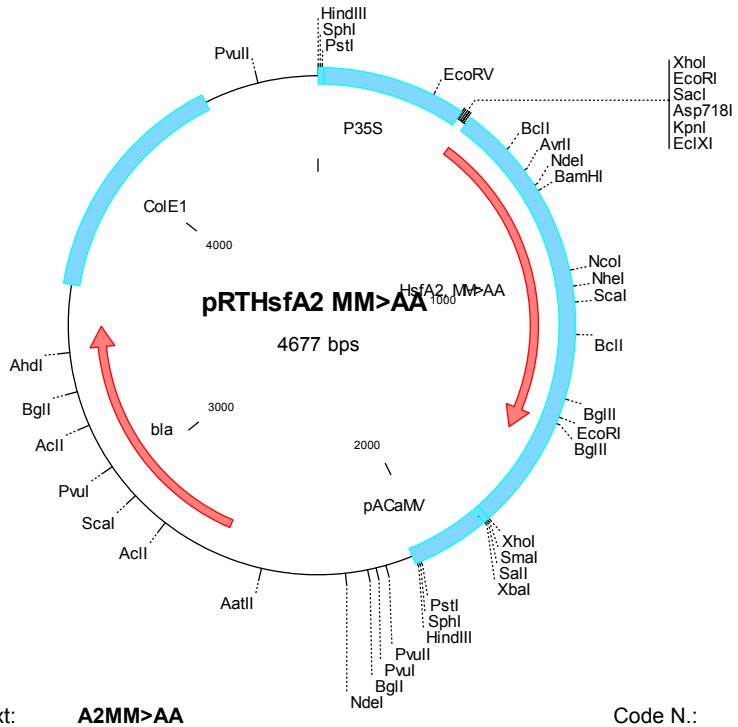
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2**

Cloning Strategy: **Fusion of pHSFT30/NotI-BamHI with OHSFT305/BamHI-NotI, NotI filled in, subcloned into pRT101/SmaI**

Used for: **Expression of HsfA2 (aa 1 to 351) in plant cells**

Constructed by/obtained from: **E. Treuter** Date:

Reference: **Treuter et al. MGG 240(93)113** Sequencing: JMT 12/91



Name in text: **A2MM>AA** Code N.:

Insert: **Lp-HsfA2 (HR-B:MM193/4>AA cDNA** **RC 72**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Host Organisms: Functional Markers:

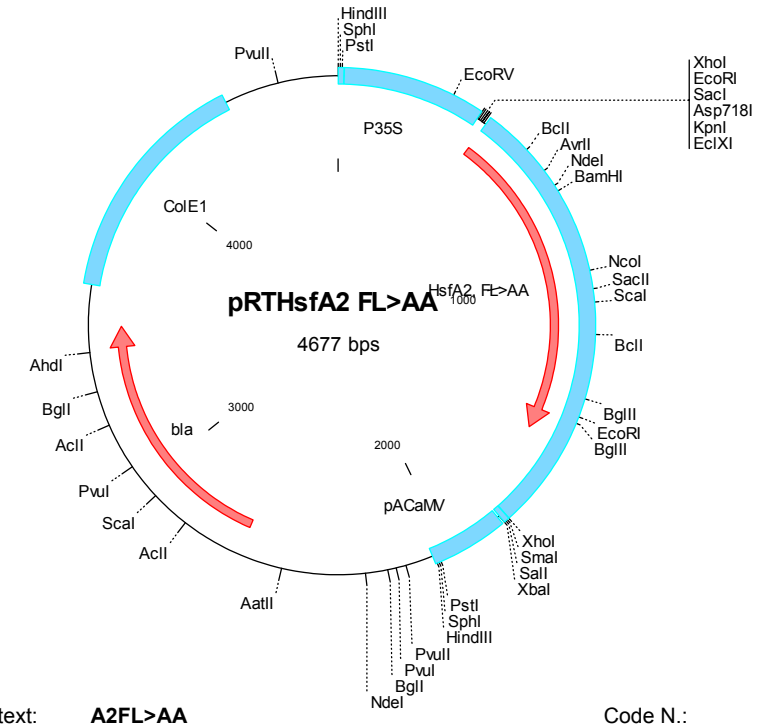
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2 MM>AA**

Cloning Strategy: **Ligation ofNcoI/XbaI fragment of pCJ309AD into pRTHsfA2 cut NcoI/XbaI**

Used for: **Expression of HsfA2 (aa1-351, FL193/4>AA) in plant cells**

Constructed by/obtained from: **R. Calligaris** Date: **04/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC153**



Name in text: **A2FL>AA** Code N.:

Insert: **Lp-HsfA2 (HR-B:FL196/7>AA cDNA** **RC 73**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Host Organisms: Functional Markers:

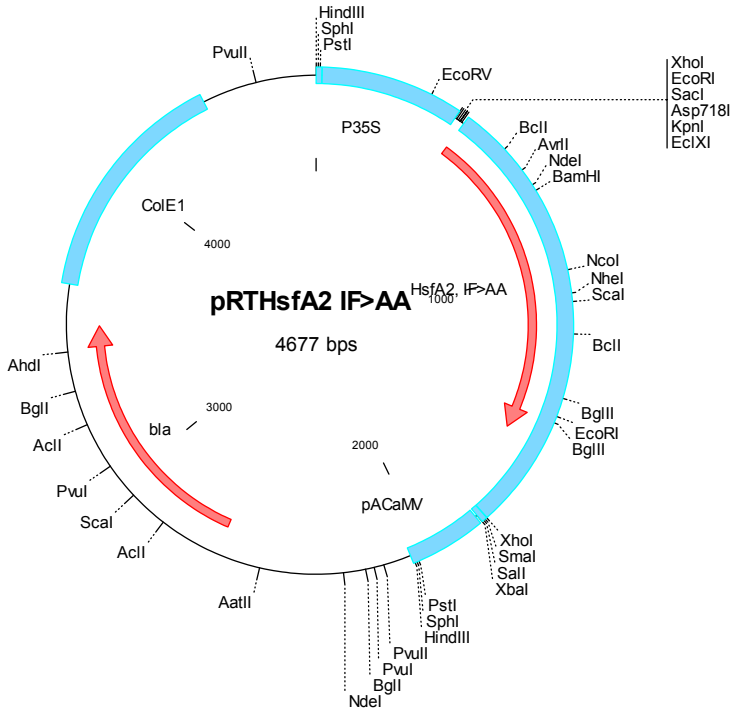
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2 FL>AA**

Cloning Strategy: **Ligation ofNcoI/XbaI fragment of pCJ310AD into pRTHsfA2 cut NcoI/XbaI**

Used for: **Expression of HsfA2 (aa1-351, FL196/7>AA) in plant cells**

Constructed by/obtained from: **R. Calligaris** Date: **04/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC154**



Name in text: **A2IF>AA** Code N.:

Insert: **Lp-HsfA2 (HR-B: IF200/1>AA cDNA** **RC 74**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Host Organisms: Functional Markers:

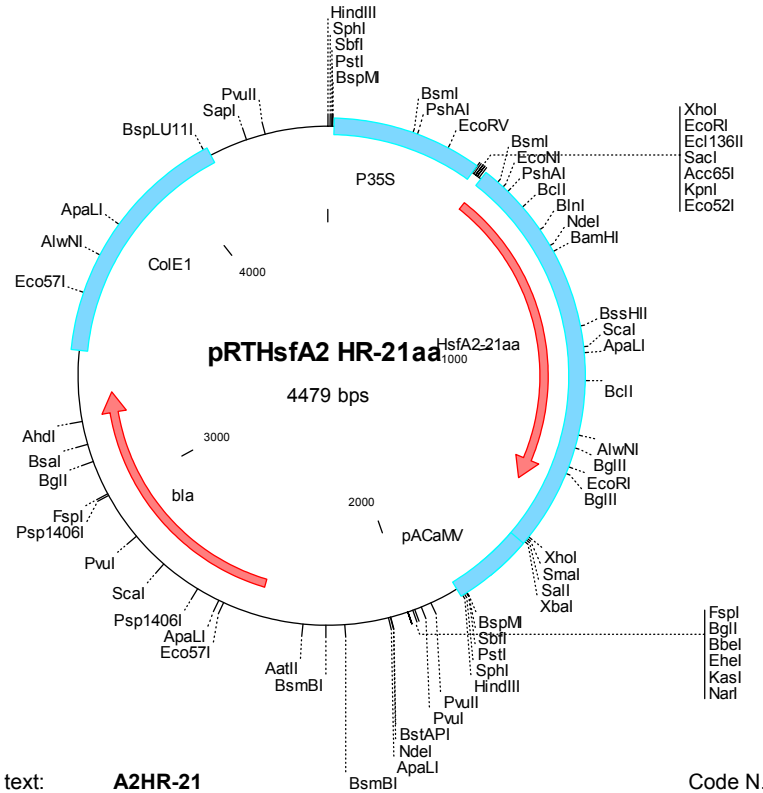
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2, IF>AA**

Cloning Strategy: **Ligation ofNcoI/XbaI fragment of pCJ311AD into pRTHsfA2 cut NcoI/XbaI**

Used for: **Expression of HsfA2 (aa1-351, IF200/1>AA) in plant cells**

Constructed by/obtained from: **R. Calligaris** Date: **04/2000**

Reference: **Laborbuch** Sequencing: **RC155**



Name in text: **A2HR-21** Code N.:

Insert: **Lp-HsfA2 (HR -21 aa) cDNA** **RC 77**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Host Organisms: Functional Markers:

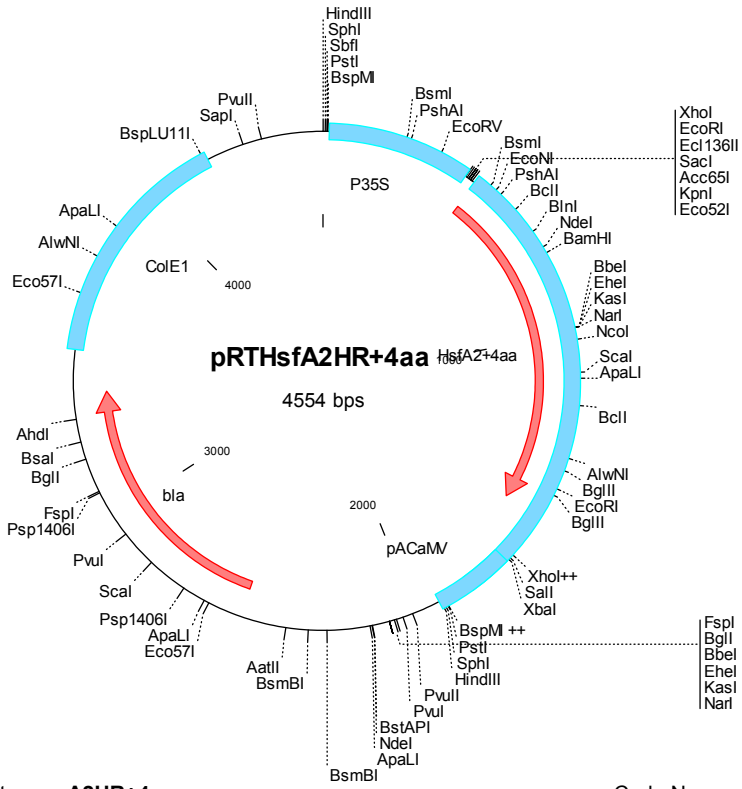
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2 MM>AA**

Cloning Strategy: **Ligation of BamHI/XbaI fragment of pADHsfA2dN95 HR -21aa (RC67) into pRTHsfA2 cut BamHI/XbaI**

Used for: **Expression of HsfA2 (aa1-168, 192-351) in plant cells**

Constructed by/obtained from: **R. Calligaris** Date: **05/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC158**



Name in text: **A2HR+4** Code N.:

Insert: **Lp-HsfA2 (HR +4 aa) cDNA** **RC 78**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Host Organisms: Functional Markers:

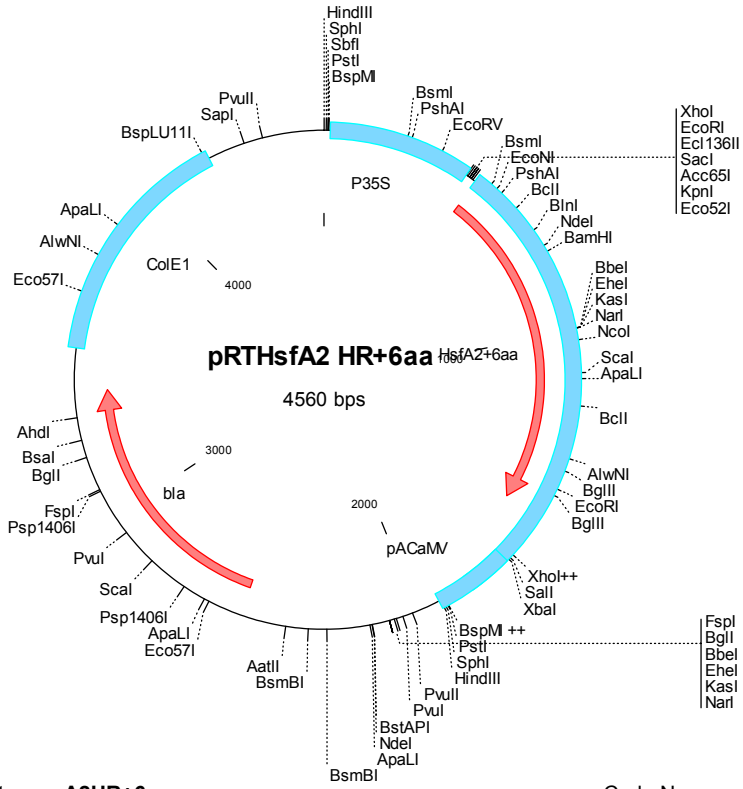
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2 MM>AA**

Cloning Strategy: **Ligation of BamHI/XbaI fragment of pADHsfA2dN95 HR+4aa (RC68) into pRTHsfA2 cut BamHI/XbaI**

Used for: **Expression of HsfA2 (aa1-351, at Q168 + ARGA) in plant cells**

Constructed by/obtained from: **R. Calligaris** Date: **05/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC159**



Name in text: **A2HR+6** Code N.:

Insert: **Lp-HsfA2 (HR +6 aa) cDNA** **RC 79**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Host Organisms: Functional Markers:

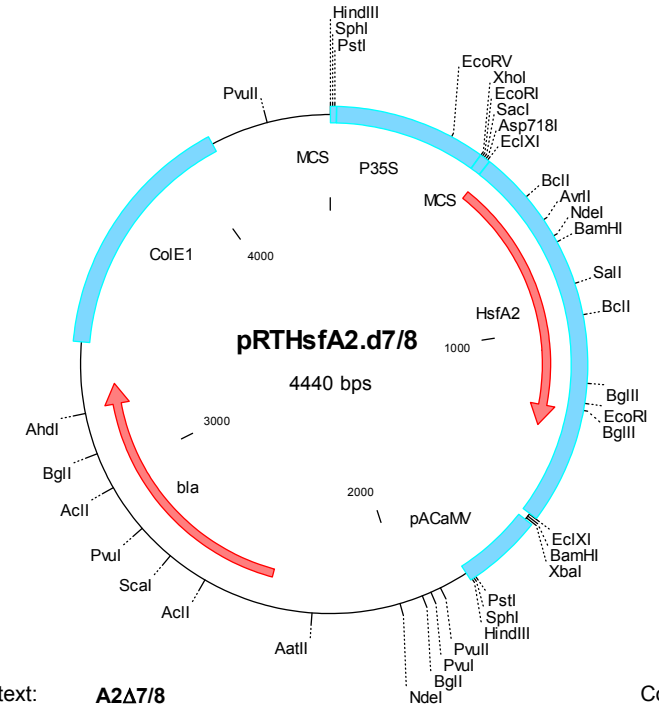
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfA2 MM>AA**

Cloning Strategy: **Ligation of BamHI/XbaI fragment of pADHsfA2dN95 HR+6 aa (RC69) into pRTHsfA2 cut BamHI/XbaI**

Used for: **Expression of HsfA2 (aa1-351, at Q168 + ARGVGA) in plant cells**

Constructed by/obtained from: **R. Calligaris** Date: **05/2000**

Reference: **Laborbuch, Promotion** Sequencing: **RC160**



Name in text: **A2Δ7/8** Code N.:

Insert: **Lp-HsfA2Δ7-8 cDNA,SalI** **ET 738/9**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Synonyms: **pEHSF30.d7-8, pRT30.d7-8**

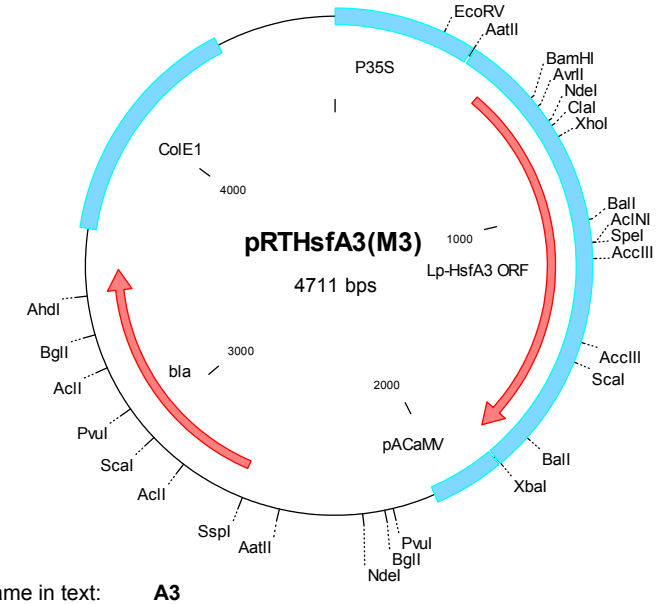
Host Organisms:	Functional Markers:
1. **E. coli**	**Amp^r**
2. **Plant cells**	**HsfA2.d7-8 (aa1-136,VD,213-351)**
	SalI site (aa 137/138)

Cloning Strategy: **Introduction of SalI by site directed mutagenesis in pBSE30S, recloning via XhoI-XbaI into pRT101; deletion of HR-A/B region between aa 136 and 213**

Used for: **Expression in plant cells**

Constructed by/obtained from: **E. Treuter** Date:

Ref.: **Promotion** Sequencing: **WS 120/2+3**



Name in text: **A3** Code N.:

Insert: **Lp-HsfA3(M3) cDNA** **K 8, K 9**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

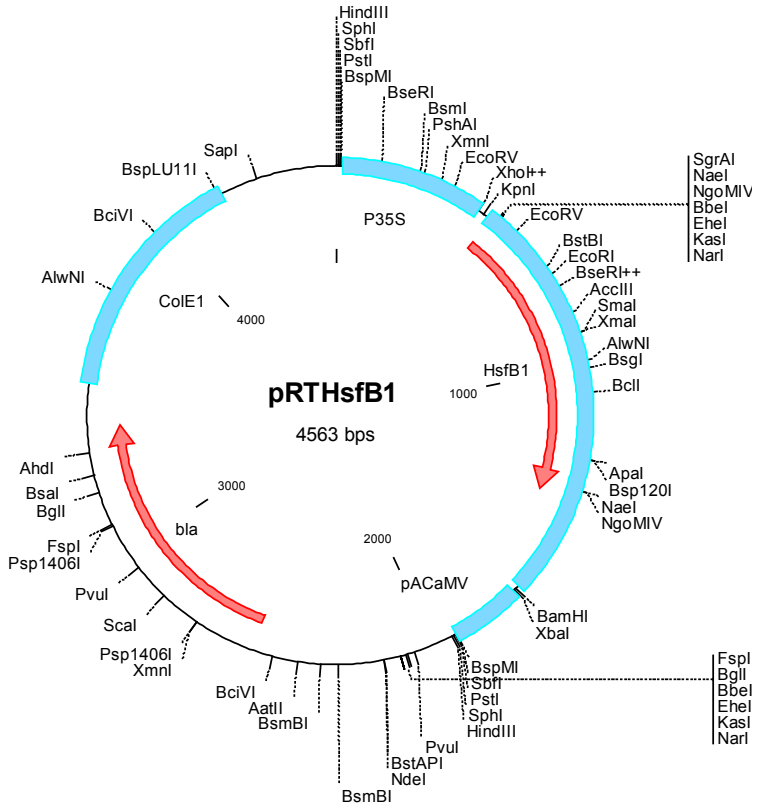
Host Organisms:	Functional Markers:
1. **E. coli**	**Amp^r**
2. **Plant cells**	**HsfA3**

Cloning Strategy: **PCR amplification of A3 cDNA fragment from A3full(pBSK) using 5' primer GSP19 (SalI) and 3' primer GSP20 (XbaI); insertion of SalI/XbaI fragm. into XhoI/XbaI cut pRT101;**

Used for: **Expression of Lp-HsfA3 (M3, aaM86-F508) in plant cells**

Constructed by/obtained from: **E. Schmidt** Date:

Reference: **Laborbuch** Sequencing: **Se 115,116**



Name in text: **B1** Code N.:

Insert: **Lp-HsfB1 cDNA** **ET 40**

Cloning Vector: **pRT101** (Töpfer et al. NAR 15(87)5890)

Synonyms: **EHSF1.2437S, pRT24S**

Host Organisms: Functional Markers:

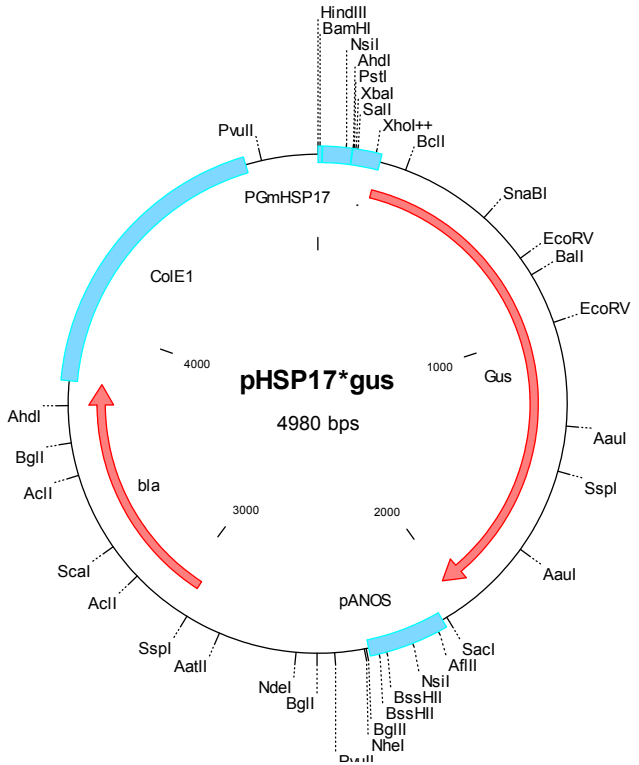
1. **E. coli** **Amp^r**
2. **Plant cells** **HsfB1**

Cloning Strategy: **Fusion of pHSFT37/NotI-EcoRI with OHSFT24/EcoRI-NotI, NotI sites filled in, subcloned into pRT101/SmaI**

Used for: **Expression of HsfB1 (aa 1 to 301) in plant cells**

Constructed by/obtained from: **E. Treuter** Date:

Reference: **Treuter et al. MGG 240(93)113** Sequencing:



Name in text: **phsp17*gus reporter gene for plant cells** Code N.:

Insert **Hsf-dep. Hsp17*xgusxNOS term. cassette** **ET 444**

Cloning Vector: **pBI2**

Host Organisms: Functional Markers:

1. **E. coli** **Amp^r**
2. **Plants** ß-Glucuronidase

Cloning strategy **Gm-HSP17promoter(-254 to -159)-(-45d)35S x Ec-GUS x pANOS in pUC19 ; pHSP17gus/TaqI-fragment cloned into pBI2**

Used for: **Hsf-dep. gus reporter for plant cells**

Constructed by/obtained from: Date:

Reference: **Laborbuch** Sequencing: **GE1_285.Seq (01.02.00)**

Acknowledgments

I would like to give thanks to all those who directly and indirectly contributed to the realization of this work and unforgettable experience in Germany.

My sincere thanks to Prof. Dr. Lutz Nover for introducing me to the “plant heat stress response” field, for his supervision during the PhD work and for making this working/studying “experience” possible.

I thank Dr. Klaus-Dieter Scharf for his help, for his constant advice and critical discussions throughout this period.

I also thank Prof. Dr. Christopher Forreiter for helpful discussions and encouragement.

I express my gratitude to Mr. Christian Scharf for invaluable support in collecting MALDI-TOF and N-terminal sequencing data and for helpful advice, and to Mrs. Christina Janista for her contributions during the first stages of the project about “the oligomerization domain of HsfA2”.

I thank all past and present coworkers in the Nover`s group. Thanks to all the members of Lab. 320, in particular Mrs. Daniela Bublack for being a constant and valuable presence and for sharing her technical experience. The work would have not been possible without their contributions and support.

Curriculum vitae

Name:	Raffaella Calligaris
Date of Birth:	14.05.1968
Nationality	Italian
Address:	Via D'Annunzio 3, 34015 Muggia (TS), Italy
Status:	Married
Languages:	Italian, English, Spanish and German

Academic record

1982-1984	Liceo classico Dante Alighieri, Trieste, Italy.
1984-1987	United World College of the Adriatic, Duino (TS), Italy
1987	International Baccalaureate Diploma – United World College of the Adriatic, Duino (TS), Italy.
1987-1994	Faculty of Biological Sciences, University of Trieste, Italy.
1994	Laurea in Biological Sciences, with biochemical specialization at the University of Trieste, Italy. Thesis title: "Expression, purification and functional characterization of the human GATA-1 transcription factor". Thesis Supervisor: Profs V. Giancotti and C. Santoro.
1994-1996	Researcher, Laboratorio Nazionale CIB, Padriciano 99, AREA Science Park, Trieste, Italy.
1996-1999	PhD Scholarship at the Graduiertenkolleg: "Proteinstrukturen, Dynamic und Fuktion" at the J. W. Goethe – Universitaet, Frankfurt am Main, Dept. of Molecular Cell Biology, Botanical Institute, under the supervision of Prof. Dr. Lutz Nover.
1996-2000	PhD work under the supervision of Prof. Dr. Lutz Nover. Thesis title: "Protein interactions of heat stress transcription factors from *Lycopersicon peruvianum*".

Frankfurt am Main, May 2001