ROLE OF HEAT STRESS TRANSCRIPTION FACTOR HSFA5 AS SPECIFIC REPRESSOR OF HSFA4
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Unlike other eukaryotes, plants posses a complex family of heat stress transcription factors (Hsf) with usually more than 20 members. Among them Hsfs A4 and A5 form a group distinguished from other Hsfs by structural features of their oligomerization domains and by a number of conserved signature sequences. We show that A4 Hsfs are potent activators of heat stress gene expression, whereas A5 Hsfs act as specific repressors of HsfA4 activity. The oligomerization domain of HsfA5 alone is necessary and sufficient to exert this effect. Due to the high specificity of the oligomerization domains, other class A Hsfs are not affected. Pull-down assay and yeast two hybrid interaction tests demonstrate that the tendency to form HsfA4/A5 heterooligomers is stronger than the formation of homooligomers. The specificity of interaction between Hsfs A4 and A5 was confirmed by bimolecular fluorescence complementation (BiFC) experiments. The major role of the representatives of the HsfA4/A5 group, which are not involved in the conventional heat stress response, may reside in cell-type specific functions connected with the control of cell death triggered by pathogen infection and/or reactive oxygen species.

Hsfs have a modular structure (Fig. 1A) with an N-terminal DNA binding domain, an adjacent domain with heptad hydrophobic repeats (HR-A/B) involved in oligomerization, a cluster of basic amino acid residues essential for nuclear import [nuclear localization signal, (NLS)] and a C-terminal activation domain, which is frequently characterized by a nuclear export signal (NES) and short peptide motifs (AHA motifs) essential for the activator function (12, 10, 13, 14). Three classes of plant Hsfs (classes A, B and C) are defined by peculiarities of their HR-A/B regions (6, 8).

Unfortunately, our understanding of the complexity of the plant Hsf family is very limited. Detailed investigations on the structural and functional diversification so far are restricted to Hsfs A1, A2, A3, A9 and B1 (10, 11, 13–24, 38). An interesting new aspect of functional diversification of Hsfs was reported, when a mutant of rice (spl7) with spontaneous necrotic lesions in mature leaves was identified as a point mutation with a Trp>Cys substitution in the N-terminal part of the DNA binding domain of HsfA4d. Although the molecular consequences of this mutation need to be elaborated, the defect of HsfA4d evidently made leaves hypersensitive to small doses of stress that resulted in uncontrolled apoptosis (25). Examples for such daily stress effects on leaves are certainly connected with high light intensities and the generation of reactive oxygen species. In support of this interpretation, Davletova et al. (26) showed that transgenic Arabidopsis plants expressing a dominant negative form of HsfA4a were inhibited in early signal transduction events connected with high light and oxidative stress.

Based on details of so-called signature sequences, Hsfs A4 are closely related to Hsfs A5. Both together form a group which is clearly separated from other members of the Hsf family (8). The crucial similarities include details of the DNA binding and the oligomerization domains as well as conserved sequence motifs in the C-terminal domains (see the block diagrams in Figs. 1A, 6A and the compiled data in
supplemental materials Fig. S1 and Table S1). In this manuscript, we present data on the functional interaction of Hsfs A4 and A5, which is based on an unprecedented specificity of their oligomerization domains. Based on results from reporter assays in mesophyll protoplasts, we show that HsfA4 represents an activator of hs gene expression, whereas HsfA5 specifically inhibits HsfA4 activity. Evidently, HsfA5 forms heterooligomers with HsfA4 and hence interferes with its DNA binding capacity. The biological implications of the co-operation between Hsfs A4 and A5 will be discussed.

Experimental Procedures

General reagents and procedures - Standard protocols were used for cloning and nucleic acid analysis (27, 28). Total RNA was prepared from plant tissues by using the RNeasy® Plant mini kit (Qiagen, Hilden, Germany). For cDNA synthesis Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas, St. Leon-Rot, Germany) was used according to the manufacturer’s protocol. PCR fragments for subcloning were generated by using the High Fidelity PCR Enzyme Mix (Fermentas).

Protein extraction, SDS polyacrylamide gel electrophoresis (PAGE), and protein blotting analysis were performed as described (19, 29). The generation and use of specific antisera against individual tomato (Lycopersicon esculentum) Hsfs (HsfA1, A2, A3) and Hsp17-CI were described before (12, 21, 29). Primary antibodies for immunodetection of green fluorescent protein (GFP), myc, HA, and strep -tagged proteins were obtained from Roche Diagnostics (Mannheim, Germany), HISS Diagnostics (Freiburg, Germany), and IBA (Göttingen, Germany). Horse radish peroxidase (HRP) -conjugated secondary antibodies were obtained from Sigma-Aldrich (Taufkirchen, Germany).

For transient gene expression studies, tobacco (Nicotiana plumbaginifolia) leaf mesophyll protoplasts were used. Polyethylene glycol (PEG)-mediated co-transformation of reporter and Hsf expression plasmids was used. The corresponding sequences for affinity tags (31, 32). Further deletions or modifications were done on the basis of these parental expression vectors. An overview of all constructs and primer sequences are compiled in Table S2. For subcellular localization studies, PCR fragments of the corresponding Hsfs were subcloned into p35dS::GFP to generate in-frame GFP-Hsf fusions. The binary BiFC plant transformation vectors pSPYNE and pSPYCE (33) were kindly provided by Klaus Harter (CPMB, University of Tübingen, Germany) and were used as template DNA for PCR amplification of myc-YN and HA-YC encoding sequences for cloning into pRT vectors in order to achieve compatible cloning sites to create Hsf-myc-YN and Hsf-HA-YC fusion constructs (see Supplemental Table S2).

Expression constructs for yeast and E. coli - For expression of tomato HsfA4b-Strep in yeast cells, the corresponding DNA fragment was subcloned from a plant expression construct into pAD5Δ (16). Expression constructs for GST-pull-down bait proteins were based on plasmid pGEX-4T-1 (Amersham Biosciences, Freiburg, Germany) and generated by in-frame fusions of DNA fragments encoding C-terminal parts of tomato Hsfs A4b (aa residues 112-393) and A5 (aa residues 110-478), respectively. Full length HsfA1 and 3HA-HsfA5 encoding sequences were cloned in pJC vectors (22).

GST pull-down interaction assay - Bait proteins were purified with the GST Purification Module (Amersham Biosciences) according to the manufacturer’s protocol. The pull-down assay was conducted in 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.01% NP40 (supplemented with Complete™ Protease Inhibitor, Roche Diagnostics) by incubation of GST-Hsfs bound to Glutathion-Sepharose with lysates prepared either from yeast cells expressing HsfA4b-Strep...
or from *E. coli* cells expressing HsfA1 or 3HA-HsfA5, respectively. The bound proteins were eluted and separated by SDS-PAGE and detected with the appropriate antibodies by protein blot analysis.

**Two-hybrid interaction assay** - For two hybrid interaction studies the pGal4-BD bait and pGal4-AD prey vector system (Stratagene) was used as described previously (10, 21). All Arabidopsis Hsf fusion constructs used in this study were described earlier (14) and were kindly provided by Pascal von Koskull-Döring (Goethe-University, Frankfurt). The strength of interaction was confirmed by colony growth in presence of the histidine biosynthesis inhibitor 3-aminotriazole (3AT).

**Intracellular localization and interaction studies** - Tobacco protoplasts transformed with appropriate combinations of plasmids encoding chimeras of Hsfs with GFP or with YFP domains were analysed after 16 h of expression. For nuclear retention of Hsf proteins, protoplasts were incubated in presence of 20 ng ml<sup>-1</sup> leptomycin B (LMB, kindly provided by Minoru Yoshida, Tokyo) added 3 h before harvesting (13). Fluorescence microscopy analysis of protoplasts transformed with GFP-Hsf fusion constructs was performed immediately after harvesting without any further treatments. Fluorescence light emission of recombined YFP in heterooligomeric Hsf-YN/Hsf-YC complexes was determined after fixation of protoplasts and staining with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) to visualize the nuclei (13).

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

**Results**

**Transactivation potentials of HsfA4 and HsfA5** - As starting point, we studied the transactivation potential of tomato Hsfs A4b and A5 (Fig. 1A) using transient expression assays with tobacco mesophyll protoplasts and two types of reporter genes: (i) plasmid borne GUS reporter gene harboring a 1 kb upstream region of the soybean *Hsp17.3B* gene (34) and (ii) endogenous chromatin-embedded Hsp17 genes, which comprise a set of Hsf dependent genes encoding the members of Hsp17-CI and Hsp17-CII protein classes (35, 29). Because of the excellent antisera available for proteins of the class CI members, we present here only the data obtained for this group of proteins. With few exceptions, results obtained for the two types of reporters were very similar.

In Fig. 1 B, the GUS activity observed in a sample transformed with the reporter plasmid only represents the basal level expression due to the endogenous Hsf cocktail present in tobacco protoplasts (marked with dotted line). GUS activities above this level correspond to the transactivation potential of the transiently expressed Hsfs. Expression of HsfA4b alone (sample 1) showed nearly 40 fold stimulation of reporter gene expression as compared to the basal level, whereas expression of HsfA5 showed no activity (sample 8). In agreement with the GUS activities, expression of endogenous Hsp17 genes was high in sample 1 but not detectable in sample 8 (Fig. 1C).

The contrasting behavior of the two Hsfs in reporter assays was surprising as both share very similar basic structural features, particularly of their DNA binding domains and of their C-terminal domains containing the activator motifs (see block diagram in Fig. 1A and Table S1). To examine any functional interaction between them, we co-transformed tobacco protoplasts with a constant amount of HsfA4b and increasing amounts of HsfA5 expression plasmids (Fig. 1B, samples 2 to 7). Two effects were observed: (i) The level of HsfA4b increased as a result of co-expression with HsfA5. (ii) In contrast to the increased HsfA4b accumulation, the GUS reporter activity decreased in direct correlation with increasing HsfA5 expression (see Hsf expression control and GUS activity in samples 2 to 7 respectively in Fig. 1B). Similar effects of HsfA5 on HsfA4b activity were observed on the endogenous Hsp17 level as reporter (Fig. 1C, samples 1 to 7). Interestingly, the repressor effect of HsfA5 was more pronounced on the chromatin-embedded Hsp17 than on the plasmid borne GUS reporter gene (compare results with samples 1 and 5 in Figs. 1B vs. C).

Protein blot analysis (Hsf expression control, Fig. 1B) showed that both HA-tagged Hsfs accumulated to detectable levels. Usually, the level of HsfA5 was higher than that of HsfA4b, ruling out the simple explanation that low expression of HsfA5 was the cause for its lack of activity. We conclude that HsfA4b is a
strong activator, whereas HsfA5 has no activator potential on either plasmid borne or chromatin-embedded reporters.

Next, we examined whether the repressor effect of HsfA5 is specific for HsfA4b or HsfA5 functions as a general repressor of Hsf activity, e.g. by competing for DNA binding sites. To this aim, we co-expressed HsfA5 together with other well characterized members of the tomato Hsf family, i.e. Hsfs A1, A2 and A3 (Fig. 1D). All three Hsfs are potent transcriptional activators (samples 9, 11, 13). However, the activity of none of them was repressed in the presence of HsfA5, but was rather increased (Fig. 1D, samples 10, 12, 14). We conclude that the HsfA5 function as repressor is highly specific for HsfA4b and does not simply result from competition for Hsf binding sites.

As an additional proof for the selectivity of HsfA5 repressor function, we expressed HsfA5 and examined the hs response of the tobacco protoplasts following temperature treatment as indicated in Fig. 2A. The accumulation of Hsp17 in the mock transformed protoplasts (Fig. 2B) reflects the hs-induced activity of the tobacco Hsf system. It was completely blocked by the expression of a dominant negative form of tomato HsfA1, i.e. HsfA1ΔC394, which competes for DNA binding but has no activator function. In contrast to this, expression of HsfA5 had no detectable effect. Evidently, the hs-induction in these mesophyll protoplasts completely depends on the activity of A1-type but not on A4-type Hsfs. Furthermore, HsfA5 does not act as a general repressor. These results nicely confirm the fundamental differences between Hsfs A1, A2, and A3 on one hand and Hsfs A4/A5 on the other.

**HsfA5 effectively binds to heat stress elements:**

The inactivity of HsfA5 prompts the question about the functionality of its DNA binding domain. Hence, we studied DNA binding by using a repressor reporter assay (15) that allows *in vivo* assessment of DNA binding activity independent of the activator function of Hsfs. The repressor reporter contains multiple Hsf binding sites immediately downstream of the TATA box of the CaMV 35S promoter (Fig. 3A). Binding of a Hsf blocks the function of the 35S-promoter and thus diminishes transcription of the GUS gene. Depending on the expression levels and DNA binding capacities Hsfs A1, A4b and A5 strongly reduced the detectable GUS activity (Fig. 3B, samples 2 to 8).

Reference points are sample 1 showing GUS activity in the absence of transiently expressed Hsfs and sample 9 expressing a DNA binding mutant form of HsfA5. We further confirmed the functionality of the HsfA5 DNA binding domain by testing in fusion proteins with the C-terminal activation domains of HsfA2 or HsfA4b respectively. As expected, both hybrid Hsfs behaved as strong transcriptional activators (data not shown).

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

In order to define the structural elements of HsfA5 required for the repression of HsfA4b activity, we prepared a series of deletion forms (Fig. 4A, constructs b to c) and a DNA binding mutant form (construct f) and tested them with respect to their repressor activity in transient reporter assays (Fig. 4B, E). As expected from the previous results (Fig. 1B), none of the HsfA5 mutant forms showed any activity on their own (data not shown), but as long as they contained the oligomerization domain (OD) they effectively repressed HsfA4b activity (Fig. 4B, samples 4, 7, 8). Note that the smallest HsfA5 fragment with full repressor activity contained only the OD (construct c). Evidently, the oligomerization domain of HsfA5 is necessary and sufficient to exert the repressor effect on HsfA4b. In support of this, HsfA5 fragments lacking the OD (Fig. 4A, constructs c and d) did not repress HsfA4b activity (Fig. 4B, samples 5, 6).

DNA binding and transcriptional activation by Hsfs are strongly dependent on their oligomerization state. Hsf deletion mutants lacking their OD are poor in DNA binding and activator function (16). The repressor reporter assay described above allowed us to test, if the DNA binding activity of HsfA4b could be affected by co-expression with HsfA5 or its mutant forms (Fig. 4C). The effects observed can be summarized as follows: (i) As shown before (Fig. 3B), HsfA4b blocked the GUS expression (Fig. 4C, sample 2), and co-expression of HsfA5 enhanced this effect (sample 3). This enhancement is mainly due to the marked increase of the HsfA4b level in the presence of HsfA5 (Fig. 4D, sample 3) (ii) In combinations of HsfA4b with HsfA5 mutant forms containing the OD (samples 4, 7, 8), GUS expression was much less diminished than in sample 3. Evidently, interaction with the truncated forms of HsfA5 affected the DNA binding affinity of HsfA4b. (iii) As expected,
The structural prerequisites of HsfA4b, i.e. functional DNA binding and oligomerization domains as well as the C-terminal activator domain with AHA motifs, are basically similar with those defined for HsfA1 and HsfA2 (18, 22). Therefore, we wanted to know, whether the repressor effect of HsfA5 could also act on HsfA1, provided the OD regions are compatible for interaction. To this aim, we tested HsfA1 w.t. and a hybrid form containing the oligomerization domain of HsfA4b (Fig. 5A). As predictable from the results shown in Fig. 1C, the expression levels of Hsp17 in samples with HsfA1 w.t. were not much affected by the presence of HsfA5 (Fig. 5B, samples 1, 2, 3). However, the high activity of the hybrid form observed in sample 4 was completely abolished in the presence of the HsfA5 fragment (Fig. 5B, samples 5, 6). Interestingly, inactivation of the hybrid HsfA1 caused by HsfA5 fragment was accompanied by a marked increase of the protein level (see protein blot analyses in Fig. 5C). Actually, the same holds true for HsfA4b in combination with HsfA5 (see expression controls in Fig. 1B, lanes 2-7). It is tempting to speculate that, similar to observations in yeast and mammalian cells, inactive transcription factors are more stable because of proteasome activities connected with active transcription complexes (36).

The activator/repressor relationship of Hsfs A4 and A5 is also found in Arabidopsis- In Arabidopsis two genes encode A4-type Hsfs, namely AtHsfA4a and AtHsfA4c (At4g18880 and At5g45710) and one gene encodes AtHsfA5 (At4g13980). The basic structure of these Hsfs is very similar to their tomato homologues (block diagrams in Fig. 6A and Table S1). Because of the relatively low activator potential of the two Arabidopsis Hsfs A4a and A4c as compared to tomato HsfA4b, we used a high affinity activator reporter (pHSE9::GUS) for these tests (Fig. 6B). The results were very similar to those for tomato Hsfs, i.e. AtHsfA5 effectively repressed the activities of both AtHsfA4a and A4c (Fig. 6B, samples 3, 6) and in the repressor reporter assay co-expression with AtHsfA5(R77D) relieved the expression block by AtHsfs A4a and A4c (Fig. 6C, samples 3 and 5). Other class A Hsfs of Arabidopsis, e.g. HsfA1a, A1b, A2 A3 etc were not affected by co-expression with AtHsfA5, and we observed cross-species inhibition between tomato and Arabidopsis Hsfs A4 and A5 (data not shown).

Physical interaction and intracellular distribution of HsfA5 and HsfA4 - Our interpretation of the repressor effect of HsfA5 on the activator function of HsfA4 implicates an unprecedented specificity of recognition among the A4/A5 group of Hsfs, which evidently excludes other members of the class A Hsfs. We wanted to support this conclusion by independent approaches. First, we tested protein interactions using yeast two hybrid system with Arabidopsis Hsfs A1a, A4a, A4c and A5 in bait and Hsfs A4c and A5 in prey positions (Fig. 7A). All yeast strains grew normally on non-selective medium (-WL), but on selective medium (-WLH and –WLH +3-AT), only strains with strong protein interactions produced colonies. These are exclusively the strains with the heterodimERIC protein interactions within the A4/A5 group (see rows 3, 5, 8, 9). As expected, no interactions were found with the Gal4-DBD alone (nos. 1 and 6) or with HsfA1a in bait position (rows 2, 7). The same is true for any other of the 19 Arabidopsis Hsfs in bait position (data not shown). Surprisingly, even the homodimeric interactions within the A4/A5 group were too weak to be detected in this system. The results clearly indicate that at least in the yeast two hybrid test, which is based on dimeric protein interactions, formation of heterooligomers between Hsfs A4 and A5 was much preferred as compared to homooligomers. These results were basically confirmed by a pull-down assay with tomato Hsfs A4b and A5 fused to glutathione-S-transferase (Fig. 7B). The signals detected from the heterologous combinations (boxed) were clearly much stronger than those from the homologous combinations. Moreover, no pull-down of HsfA1 was detectable with any of the three baits used.

To study the localization and interaction of tomato Hsfs A4b and HsfA5 in plant cells, we expressed the corresponding GFP and YFP fusion proteins in tobacco mesophyll protoplasts (Fig. 8). GFP-HsfA4b was detected in the nucleus, whereas GFP-HsfA5 was predominantly in the cytoplasm (Fig. 8A, samples 1, 2). However, after addition of Leptomycin B (LMB), an inhibitor of the...
nuclear export receptor, GFP-HsfA5 strongly accumulated in the nucleus (Fig. 8A, sample 3). This result indicates that, similar to the tomato HsfA2 (13), the NES function of HsfA5 dominates its NLS function. Although localized mainly in the cytoplasm, HsfA5 shuttles between cytoplasm and nucleus. Next, we investigated the possibility that the intracellular localization of HsfA5 might be influenced by HsfA4b. Indeed, co-expression of GFP-HsfA5 with HsfA4b-3HA caused nuclear localization of the former (Fig. 8A, sample 4). Evidently, the balance of nuclear import and export for the heterooligomers of Hsfs A4b and A5 is shifted towards the import reaction. Such nuclear retention effect of HsfA5 was not observed by co-expression with HsfA4b(ΔOD) or of Hsfs A1, A2 or A3 (data not shown).

A very stringent method to demonstrate tight physical interaction between two proteins in plant cells is the bimolecular fluorescence complementation (BiFC) analysis (33). We used fusion proteins of tomato Hsfs A4b and A5 with complementary N-terminal and C-terminal fragments (YN and YC) of the yellow fluorescent protein (YFP) (see Materials and Methods). Co-transformations of tobacco protoplasts with YN and YC fragments alone served as background control (Fig. 8B, sample 1). Protoplasts expressing HsfA4b-YN and HsfA4b-YC showed strong YFP complementation in the nucleus (Fig. 8B, sample 2). In contrast to this, samples expressing HsfA5-YN and HsfA5-YC gave extremely faint signals (Fig. 8B, sample 3). We reasoned that this could be the result of the general distribution of HsfA5 in the cytoplasm. Therefore, we co-expressed HsfA5-YN and HsfA5-YC together with HsfA4b and HsfA1 containing no fluorescent tag. Indeed, a clear nuclear YFP signal was detectable in the presence of HsfA4b (Fig. 8B, sample 4) but not of HsfA1 (sample 5). Interestingly, the nuclear detection of YFP fluorescence in sample 4 indicates the formation of (HsfA5-YN/HsfA5-YC)-HsfA4b heterotrimer or multimers of this type. As would be expected, co-expression of HsfA4b-YN and HsfA5-YC or vice versa, also resulted in strong nuclear YFP signals (Fig. 8B, samples 6, 7). However, no complementation was observed when either one of the two Hsf was lacking its oligomerization domain, e.g. Fig. 8B, sample 8. These results with the BiFC technique clearly confirm the specificity and structural requirements of the interaction between Hsfs A4b and A5.

Discussion

Compared to all other organisms with 1 to 3 Hsfs or Hsf-related transcription factors (3, 4, 5, 7), the multiplicity of members of the Hsf family in plants is striking (6, 8). Although our knowledge is still very limited, functional diversification seems to be the main reason for the coexistence of more than 20 Hsfs in plants. Remarkable cases of specialization by selective expression were reported for HsfA2 as strongly hs-induced protein (10, 11) and for HsfA9 with exclusive expression during seed maturation (20, 37, 38). On the other hand, the well studied examples of three tomato Hsfs A1, A2 and B1 impressively illustrated the extent of functional diversification and co-operation of Hsfs. All three together form a functional triad for the essential three phases of the hs response, i.e. the triggering (HsfA1 as master regulator), maintenance and high efficiency of hs gene transcription (co-operation of HsfA1/A2 heterooligomers with HsfB1) as well as for the restoration of house-keeping gene transcription during the recovery phase. For the latter co-operation of HsfB1 with putative house-keeping transcription factors is required as well (19, 22).

Compared to this, the situation with Hsfs A4 and A5 described here opens a completely novel aspect of Hsf cooperation. When tested in reporter assays with Hsf-dependent promoter constructs, tomato HsfA4b was functionally equivalent or even stronger than Hsfs A1 and A2, whereas HsfA5 was completely inactive (Figs. 1 and 2). This result was surprising, since HsfA5 has all necessary functional elements of a bona fide activator Hsf. It has a functional DNA binding domain (Fig. 4) and harbors a typical and highly conserved AHA motif in its C-terminal part shown earlier to be crucial for the activator function of Hsfs (15, 21, 18, 14, 39). Indeed, fusion of the C-terminal domain of HsfA5 to the Gal4 DBD gives a weakly active activator protein in yeast and a mutation of the Trp residue in the predicted AHA motif (\(-\text{FWEQFL} \rightarrow -\text{FAEQLF}\), Table S1) abolished this activity. These results indicate that the predicted AHA motif is functional but not in its natural context of the w.t. HsfA5 (14).

The inhibition of HsfA4 activity by HsfA5 is found for both tomato and Arabidopsis (Figs. 1 and 7 respectively) as well as for corresponding cross-species combinations of these Hsfs. Considering the conservation of Hsfs A4 and A5 (Fig. S1 and Table S1), it is tempting
to speculate that it is indeed a fundamental feature of the Hsf system in plants. The inhibitory function of HsfA5 requires only its OD. Obviously, no additional factors, e.g. a putative corepressor recruited by the C-terminal domain, are involved. In view of these results, it is also not reasonable to discuss a simple shielding mechanism for the AHA motifs of HsfA4 by a putative internal repressor domain of HsfA5. Evidently, the repressor effect mainly results from the interference with the oligomeric state of HsfA4b, which is essential for efficient DNA binding and activator functions. Consistent with this interpretation, HsfA1, which itself is insensitive to the inhibitory effect of HsfA5, was made sensitive by exchanging its oligomerization domain with that of HsfA4b. Thus, provided the compatible oligomerization domains, the repressor effect can be imparted onto other Hsfs. The whole molecular context of HsfA4 is not required (data not shown).

The stringent interaction behaviour of Hsfs A4 and A5 demonstrates an unexpected specificity generated by their oligomerization domains. Unfortunately, sequence inspection of the HR-A/B regions of HsfA1/A2-type on the one hand and HsfsA4/A5-type on the other gave no immediate clue to the basis of this specificity. Moreover, pull-down assays and yeast two-hybrid interaction tests (Fig. 7) clearly indicate that the formation of heterooligomers is preferred. Very likely, this tendency is crucial for the strong repressor effect of HsfA5 on HsfA4 activity. In all cases investigated, deletion or heterologous replacement of the oligomerization domain in one of the two partner Hsfs abolished the repressor effect.

What can be the biological implication of this pair of activator/repressor Hsfs? Are specific genes addressed by HsfA4? All our assays in this work were based on Hsf-dependent reporters, which respond equally well to HsfA1/A2 and to HsfA4. Also other reporters tested did not indicate promoter specificity (data not shown). However, this can not exclude a situation in plants, where combination of HsfA4 with other tissue-specific transcription factors creates promoter specificity not detectable in our reporter assays. This argument also holds true for the function of HsfA4 for hs-induced gene expression. The dominant role of HsfA1 as master regulator (Fig. 2) may not be valid to the same extent for all tissues and developmental stages.

An essential aspect of the discussion about the specialized function of Hsfs A4 and A5 in plants is their expression profile. From EST and whole genome sequence data bases of rice and Arabidopsis we identified, a single HsfA5 but usually one or two A4 Hsfs. All members of the HsfA5 subgroup are much more conserved among each other than the members of the HsfA4 subgroup (Fig. S1 and the sequence details compiled in Table S1). The detection of numerous ESTs indicates that representatives of the A4/A5 group are well expressed in different plant tissues. Analysis of the Arabidopsis microarray data bases confirmed that Hsfs A4a, A4c and A5 mRNAs are indeed found in many tissues, albeit at very different levels which change with the developmental stages and stress conditions (Fig. S2). Usually, the levels of HsfA5 mRNA in Arabidopsis are markedly lower than those of Hsfs A4a and A4c. This contrasts to the high abundance of HsfA5-specific ESTs in the data bases of other plants. These considerations are helpful, but it should be recalled that all data are based on RNA analyses which may not give direct information about the corresponding protein levels.

Intriguing hints for a specialized function of HsfA4 came from the analyses of a rice HsfA4d mutant showing spontaneous necrotic lesions in mature leaves due to evident hypersensitivity to mild stress challenges (25). On the other hand, transgenic Arabidopsis plants overexpressing a dominant negative form of HsfA4a had defects in their response to oxidative stress (26). It is tempting to speculate that we are actually dealing with the same HsfA4-dependent syndrome in rice and Arabidopsis, because in both cases reactive oxygen species play key roles as stressors and signals.

In summary, the most plausible mechanism for the repressor role of HsfA5 is based on its high affinity to interact with HsfA4 to generate inactive heterooligomers. It remains to be examined whether the balance between active HsfA4 homotrimers and inactive HsfA4/HsfA5 heterotrimers could be influenced by modifications, e.g. as a result of oxidative stress as nicely summarized by Miller and Mittler (43), or whether the shuttling of HsfA5 between nucleus and cytoplasm plays a role in such a regulatory mechanism. Similar to the highly selective function of HsfA9 as part of the ABA-controlled program of seed development (37, 38), the special role of Hsfs A4 and A5 may be restricted to certain developmental stages and/or to biotic or abiotic stress challenges. We
hypothesise that HsfA5 may represent a novel type of selective repressor, regulating the function of A4-type Hsfs in plants.

REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig. 1. HsfA5 as repressor of HsfA4b activator function in tobacco protoplasts. A, block diagrams representing the basic architecture of activators and the activator reporter construct. Reporter contains the soybean Gm*hsp17.3B-CI* promoter fragment fused to *GUS* gene (34). The fragment consists of the indicated combinations of heat stress elements (HSE, for details see Nover et al. 2001) and TATA box (TA). Numbers indicate the distance in base pairs. Activator Hsfs A4b (aa 1-393) and A5 (aa 1-478) with their DNA binding domain (DBD), oligomerization domain (OD, formally defined as HR-A/B), nuclear localization signal (NLS), nuclear export signal (NES) and activator motifs (AHA). SS marks
the C-terminal signature sequences (for details see Table S1). Numbers refer to the amino acid residues. Hsfs were triple-HA-tagged to facilitate their detection (depicted as black dots).

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

D, effects of HsfA5 on the activator potential of Hsfs A1, A2 and A3. GUS activities and expression control using specific antiserum against each of the indicated Hsf (see materials and methods). 0.5 µg Hsf encoding plasmids were used.

Fig. 2. HsfA5 does not inhibit the heat stress (hs) induced expression of Hsps in tobacco protoplasts. A, pictograph showing the hs regimen. Samples 1 to 3 were harvested at indicated time points. T, protoplasts were transformed with empty vector (mock) or with expression plasmids encoding HsfA1∆C394 and HsfA5 respectively. B, protein blot analysis of the whole cell extracts from indicated samples by using α-HsfA1, α-HA or α-hsp17-CI antisera.

Fig. 3. Use of the repressor reporter assay to test for DNA binding affinity. A, block diagram showing the architecture of repressor reporter containing an array of nine HSE immediately downstream of TATA-box of the 35S promoter (TA). B, GUS activities after transformation of protoplasts with the repressor reporter and the indicated amounts of Hsf expression plasmids. C, Hsf expression controls using HA-antiserum (α-HA).

Fig. 4. Structural requirements for the repressor function of HsfA5. A, block diagrams representing HsfA5 and its mutant forms used for co-transformation with HsfA4b. Wild-type HsfA5 (a), different truncation forms (b to e) and a DBD mutant, in which the invariant Arginine residue at position 72 in the HTH motif was mutated to Aspartate (R72D). Black dots mark triple HA-tag at the N-terminus used for Hsf detection. B, GUS expression levels in samples containing HsfA4b in combination with the indicated forms of HsfA5. C, repressor reporter assay with the same combination of Hsf constructs as used in B. D, Hsf expression controls for samples 1 to 8. In lane 6 the bands for HsfA4b and for the deletion form of HsfA5 coincide.

Fig. 5. The oligomerization domain of HsfA4b is sufficient to make HsfA1 sensitive to the repressor effect of HsfA5. A, block diagrams of HsfA1, HsfA1 carrying the OD (HR-A/B region) of HsfA4b and HsfA5OD fragment used as repressor. B, expression of Hsp17 in samples transformed with the indicated Hsf expression plasmids. C, expression control of Hsfs using antisera against HsfA1 and HA-tag.

Fig. 6. Arabidopsis HsfA5 specifically represses transcriptional activator potential of AtHsfs A4a and A4c. A, block diagrams depicting basic structures of the AtHsfs. For convenience, we used as repressor only the DNA binding mutant of AtHsfA5(R77D) with mutation of the same invariant Arg residue as shown before for the tomato HsfA5 (Fig. 4). Results with w.t. HsfA5 were very similar. B, C tobacco protoplasts were co-transformed with the indicated combination of expression plasmids, i.e. 0.5 µg of Hsfs A4a or A4c and 2 µg of HsfA5(R77D) either with the activator reporter pHSE9::GUS (B), or with the repressor reporter p35S::HSE9-GUS (C).

Fig. 7. Heterooligomerization prevails over homooligomerization among A4 and A5 subgroup members from Arabidopsis and tomato. A, Yeast two-hybrid interaction test using Arabidopsis Hsfs A4c and A5 as preys and A1a, A4a, A4c and A5 as baits (see Materials and Methods for details of bait and prey constructs). Abbreviations used: AD and BD are activation and DNA binding domains, respectively of the yeast transcription factor Gal4; W (Tryptophan), L (Leucine), H (Histidine) are selection markers; 3-AT is 3-aminotriazole, an inhibitor for Histidine biosynthesis used at 10 mM concentration. B, GST fusion protein pull-down assay with recombinant tomato Hsfs A4b and A5 purified from E.coli. Purified GST or indicated fusion proteins were incubated with different whole cell protein extracts expressing HsfA4b-Strep, 3HA-HsfA5 or HsfA1 (as described in Materials and Methods). The boxes indicate the samples with higher pull-down efficiencies of the baits in combinations corresponding to the formation of heterooligomeric complexes.
Fig. 8. Intracellular localization and interaction of tomato HsfA4b and HsfA5 in tobacco mesophyll protoplasts. A, images of living protoplasts transformed with indicated expression plasmids encoding GFP-tagged Hsfs. Samples 3 represents GFP-HsfA5 after LMB treatment. B, bimolecular fluorescence complementation (BiFC) using protoplasts expressing YFP domains, *i.e.* YN/YC alone or as their fusions with wild type or mutant forms of HsfA4b and HsfA5 as indicated on the top of each panel. In contrast to (A) pictures in (B) represent fixed cells. Either HsfA4b or HsfA1 was co-expressed with the homooligomeric combination of HsfA5-YN and HsfA5-YC (sample 4 and 5 respectively). Position of the nucleus was determined by DAPI staining and is depicted as dotted circle in those images where no fluorescent signal was detectable, *i.e.* 1, 3, and 5. Fluorescence signals in other samples corresponded to the nucleus and marked by arrows.
Figure 1

A  Hsf activator constructs

HsfA4b-3HA

\[\begin{array}{cccccc}
& DBD & OD & NLS & AHA & AHA \\
1 & 111 & 194 & 212 & 250 & 338 \\
2 & 393 \\
\end{array}\]

3HA-HsfA5

\[\begin{array}{cccccc}
& SNED & CLLAS & SS \\
1 & 109 & 186 & 212 & 313 & 362 \\
2 & 437 & 478 \\
\end{array}\]

Activator reporter construct

Gmhspl7.3B-CI :: GUS

B  GUS activity

\[\begin{array}{cccccc}
pHsfA4b (\mu g) & .5 & .5 & .5 & .5 & .5 & .5 & .5 & - \\
pHsfA5 (\mu g) & - & .0625 & .125 & .25 & .5 & 1 & 2 & 2 \\
\end{array}\]

Hsf expression control

\[\begin{array}{cccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 \\
HsfA5 & - & - & - & - & - & - & - \\
HsfA4b & - & - & - & - & - & - & - \\
\end{array}\]

C  Endogenous Hsp17 expression

\[\begin{array}{cccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 \\
Hsp17-CI & - & - & - & - & - & - & - \\
\end{array}\]

D  GUS activity

\[\begin{array}{cccccccc}
pHsfA5 (\mu g) & - & 2 & - & 2 & - & 2 \\
HsfA1 & 9 & 1 & 11 & 21 & 31 & 4 \\
HsfA2 & -2 & 11 & 12 & - & - & - \\
HsfA3 & -2 & 13 & 14 & - & - & - \\
\end{array}\]

Hsf expression control

\[\begin{array}{cccccc}
9 & 10 & 11 & 12 & 13 & 14 \\
HsfA5 & - & - & - & - & - \\
\end{array}\]
Figure 2

A  Heat stress regime

B  Endogenous Hsp17 expression

<table>
<thead>
<tr>
<th></th>
<th>Mock 1</th>
<th>pHsfA1ΔC394 1</th>
<th>pHsfA5 1</th>
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<td>2</td>
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<td></td>
<td></td>
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<tr>
<td>3</td>
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</tbody>
</table>

Hsf expression control

- HsfA5
- HsfA1
- HsfA1 ΔC394

- Hsp17-CI
Figure 3

A  Repressor reporter construct

B  GUS activity

C  Hsf expression control
Figure 4

A HsfA5 and mutant forms

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<td>OD</td>
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<td>c)</td>
<td>Δ1-199</td>
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<tr>
<td>d)</td>
<td>Δ110-200 (AOD)</td>
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<tr>
<td>e)</td>
<td>110-200</td>
<td></td>
<td></td>
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<tr>
<td>f)</td>
<td>R72D</td>
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B GUS activity

Activator reporter

C GUS activity

Repressor reporter

D Hsf expression control
Figure 5

A  Hsf activator constructs

HsfA1  
1  DBD  OD  NLS  AHA  527

HsfA1(A4bOD)  
1  DBD  OD  NLS  AHA  527

HsfA5OD  
1  110  200  527

B  Endogenous Hsp17 expression

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<th>pHsfA1  (0.5 µg)</th>
<th>pHsfA1(A4bOD)  (0.5 µg)</th>
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C  Hsf expression control

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**Figure 6**

**A** Arabidopsis Hsf constructs

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<td>401</td>
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<tr>
<td></td>
<td></td>
<td>SNED CLLAS</td>
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<td>423</td>
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</table>

**B** Activator reporter

**C** Repressor reporter

GUS activity

<table>
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<tr>
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<th>GUS activity</th>
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<td>Rfu x 10^3</td>
<td>Rfu x 10^3</td>
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<tr>
<td>1 2 3 4 5 6</td>
<td>1 2 3 4 5 6</td>
</tr>
</tbody>
</table>

HsfA5 (R77D) - - + + + +

HsfA4a HsfA4c HsfA4c
Figure 7

A  Yeast two hybrid assay

Bait | -WL | -WLH | -WLH +3-AT
---|---|---|---
1 | Gal4BD- | - | -
2 | -A1a | - | -
3 | -A4a | - | -
4 | -A4c | - | -
5 | -A5 | - | -

Prey: Gal4AD-A4c

Prey: Gal4AD-A5

6 | - | - | -
7 | -A1a | - | -
8 | -A4a | - | -
9 | -A4c | - | -
10 | -A5 | - | -

B  GST pull down assay

Input 0.5% | GST | GST-Hsf-CTD
---|---|---
HsfA4b | 1 | 2 | 3 | 4
HsfA5 | 1 | 2 | 3 | 4
HsfA1 | 1 | 2 | 3 | 4
Figure 8

A  pGFP-HsfA4b  pGFP-HsfA5  GFP-HsfA5 (+ LMB)  GFP-HsfA5 (+ pHsfA4b)

1  2  3  4

B  pYn  pHsfA4b-Yc  pHsfA5-Yc  pHsfA5-Yc
    +  +  +  (+ pHsfA4b)
pYc  pHsfA4b-Yn  pHsfA5-Yn  pHsfA5-Yn

1  2  3  4

pHsfA5-Yc  pHsfA4b-Yc  pHsfA4b-Yc  pHsfA5ΔOD-Yn
    +  +  +  
pHsfA5-Yn  pHsfA5-Yn  pHsfA5-Yc  pHsfA5ΔOD-Yn

5  6  7  8
**Fig. S1.** Phylogenetic relationship of Hsfs A4 and A5. The phylogenetic tree is based on amino acid sequences of the N-terminal parts (DNA binding domains and HR-A/B regions) of Hsfs and was elaborated using the Clustalx 1-8_msw and Tree view softwares. Sequence data were derived from the following database accession numbers:

At *Arabidopsis thaliana* HsfA4a (At4g18880), HsfA4c (At5g45710), HsfA5 (At4g13980); Bv, *Beta vulgaris* HsfA5 (BQ488901); Cs, *Citrus sinensis* HsfA4a (DY270414), HsfA5 (DY300016); Gh, *Gossypium hirsutum* HsfA6 (DT548305); Gm, *Glycine max* HsfA4a (TC135284), HsfA5 (BM270993); Ha, *Helianthus annuus* HsfA5 (DY932755); Hv, *Hordeum vulgare* HsfA4a (TC42448), HsfA5 (TC151027); Le, *Lycopersicon esculentum* HsfA4a (BT014619), HsfA4b (TC107140), HsfA5 (TC155271); Lj, *Lotus japonicus* HsfA4a (AP004978); Ls, *Lactuca sativa* HsfA4a (DY973605), HsfA5 (DY974369); Ms, *Medicago sativa* HsfA4a (AF494082); Mt, *Medicago truncatula* HsfA4a (TC79769), HsfA5 (TC79192); Nb, *Nicotiana benthamiana* HsfA5 (CK287755); Nt, *N. tabacum* HsfA4a (AB014484); Os, *Oryza sativa japonica* HsfA4a (AP004879), HsfA4d (AC111015), HsfA5 (AP004999); Pa, *Phaseolus aureus* HsfA4a (AY052627); Sb, *Sorghum bicolor* HsfA4a (BM322601); So, *Saccharum officinarum* HsfA5 (CA264007); St, *Solanum tuberosum* HsfA4b (BG591987), HsfA5 (TC112724); Ta, *Triticum aestivum* HsfA4d (CV766704), HsfA5 (CJ655373); To, *Taraxacum officinarum* HsfA4a (DY824833); Zm, *Zea mays* HsfA4a (X92943), HsfA5 (EE174627)

**Fig. S2.** Expression analysis of Arabidopsis Hsfs A4a, A4c and A5 based on microarray data. A, developmental series; B, abiotic stress series; C, pathogen infections, and elicitor treatments. The color code for the signal intensities is given at the bottom. Shown are the gcRMA normalized and averaged signal intensities (http://www.weigelworld.org/resources/microarray/AtGenExpress/) visualized as “heat maps” (with GeneSpring Version 7.2, Silicon Genetics) with re-transformed linear signal intensities for Hsfs A4a, A4c, and A5. Ubiquitin 11 mRNA levels were used as constitutive expression control.
### Table S1: Sequence alignment of C-terminal signature sequences of HsfA4 and HsfA5 ¹)

<table>
<thead>
<tr>
<th></th>
<th>HsfA4</th>
<th>NLS</th>
<th>AHA</th>
<th>NES</th>
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<tbody>
<tr>
<td>1.</td>
<td>LeA4b  (TC107140)</td>
<td>ASRKRRLL 113aa NDVFQWQFLTEPQGCTEPQQVENKGINE 12aa YWNNRGVNLNMLAEMGHLSSPATGS*</td>
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<tr>
<td>2.</td>
<td>StA4b  (CV498961)</td>
<td>KRRLLV 136aa NDVFQWQFLTEPQGCTEPQVENKGINE 12aa YWNNSGYVNLNMLAEMGHLSSPATG</td>
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<tr>
<td>3.</td>
<td>AtA4c  (At5g45710)</td>
<td>HRRKRRFF 83aa NDDFQWQLEPQGCTEPQVENKGE 12aa YWNNSGVNNIT-------EKAS*</td>
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<tr>
<td>4.</td>
<td>AtA4a  (At4g18880)</td>
<td>NERKRRFP 127aa NGFWQFQFFQPSTQPQVENQGE 12aa CWWNSRNVNAITEQ-LGHLTSSERS*</td>
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<tr>
<td>5.</td>
<td>LeA4a  (BT014619)</td>
<td>NERKRRLP 130aa NGFWQFQFFQPSTQPQVENQGE 12aa FWWRKTISLQTE-LGHLTPAE*</td>
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<td>6.</td>
<td>NtA4a  (AB014484)</td>
<td>NDRKRRLP 132aa NGFWQFQFFQPSTQPQVENQGE 12aa FWWNKSNNLAEQ-LGHLTPAEKT*</td>
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<td>7.</td>
<td>HaA4a  (BQ966974+915996)</td>
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<td>8.</td>
<td>CsA4a  (CX673366)</td>
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<td>9.</td>
<td>InA4a  (CJ759462)</td>
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<td>10.</td>
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<td>11.</td>
<td>GmA4c  (AW185379)</td>
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<td>12.</td>
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<td>Amino acid sequences were derived from the EST databases (see also legend to Figs. 1 and S1) and the corresponding accession numbers are given in brackets.</td>
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<tr>
<td>Ac, <em>Agrostis capillaris</em>; Af, <em>Aquilegia formosa</em>; In, <em>Ipomoea nil</em>; for abbreviations of other plant names see legend to Fig. S1.</td>
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<tr>
<td>B: HsfA5</td>
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<td>CLLLAS</td>
<td>AHA</td>
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<td>1. LeA5 (TC115271)</td>
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<tr>
<td>7. MtA5 (TC79192)</td>
<td>YNKKRRLP 52aa STQGSNED 72aa CQLNLTLAS 74aa NDVWFENFLTERPGCSNDEEASS 23aa KNMDNQLTL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. ZmA5 (DT938817)</td>
<td>truncat. STQSSHED 54aa CHLNLSLAS 84aa NDKFWEQFLTERPGCPEAEEASF 20aa RDMGQLKL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. OsjA5 (AP004999)</td>
<td>FNKKRRLP 54aa STQSSNED 54aa CHLSLTLAS 75aa NDKFWEQFLTERPGCSETEEASS 24aa EDVEQKL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. SbA5 (AW680707+680643)</td>
<td>FHHKRRLP 54aa STQSSHED 54aa CHLNLSLAS 83aa NDKFWEQFLTERPGCSEEAEEASS 20aa RDMGQLKL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. SoA5 (CA175344+109762)</td>
<td>truncat.&gt;50aa STQSSHED 54aa CHLNLSLAS 82aa NDKFWEQFLTERPGCSEEAEEASS 19aa RDMGQLKL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. HvA5 (TC151027+ BQ759144)</td>
<td>LHKKRKLS 54aa STQSSHED&gt;50aa CHLSLTLAS 67aa NDNFWEQFLTERPGDASSGPRDP 15aa EATEQKL*</td>
<td></td>
<td></td>
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</tbody>
</table>
**Table S2 – Overview of Hsf expression constructs and primers for DNA amplification.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Construct</th>
<th>Forward (fw) and reverse (re) primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pRTdS-HsfA4b-3HA</td>
<td>fw <em>(NcoI)</em> 5’-gcataagctatgacccatctgga-3’ and re <em>(NheI)</em> 5’-gaatcagctatgacccatctgga-3’</td>
</tr>
<tr>
<td>2</td>
<td>pRTdS-HsfA5</td>
<td>fw <em>(KpnI)</em> 5’-ctcgagttcatcattgacccatctgga-3’ and re <em>(SalI)</em> 5’-ggctacaggtacctatgacccatctgga-3’</td>
</tr>
<tr>
<td>3</td>
<td>pRTdS-HsfA5 (Δ1-109)</td>
<td>fw <em>(KpnI)</em> 5’-ctcgagttcatcattgacccatctgga-3’ and re <em>(SalI)</em> 5’-ggctacaggtacctatgacccatctgga-3’ ligation via <em>KpnI</em> and <em>SalI</em></td>
</tr>
<tr>
<td>4</td>
<td>pRTdS-HsfA5 (Δ1-199)</td>
<td>fw <em>(KpnI)</em> 5’-ctcgagttcatcattgacccatctgga-3’ and re same as for No. 3 ligation via <em>KpnI</em> and <em>SalI</em></td>
</tr>
<tr>
<td>5</td>
<td>pRTdS-HsfA5 (Δ110-200)</td>
<td>fw <em>(KpnI)</em> 5’-ctcgagttcatcattgacccatctgga-3’ and re <em>(SalI)</em> 5’-ggctacaggtacctatgacccatctgga-3’ ligation via <em>KpnI</em>, <em>SalI</em>, and <em>NheI</em></td>
</tr>
<tr>
<td>6</td>
<td>pRTdS-GFP-HsfA4b-3HA</td>
<td>fw <em>(BglII)</em> 5’-taattccctggaagcggacctata-3’ and re same as for No. 3 ligation via <em>BglII</em> and <em>XbaI</em></td>
</tr>
<tr>
<td>7</td>
<td>pRTdS-GFP-HsfA5</td>
<td>fw <em>(SacI)</em> 5’-gcctggaagcggacctata-3’ and re same as for No. 3 ligation via <em>SacI</em> and <em>XbaI</em></td>
</tr>
<tr>
<td>8</td>
<td>pRTdS-myc-YN</td>
<td>fw <em>(NheI)</em> 5’-ggaatcaggtacctatgacccatctgga-3’ and re <em>(XbaI)</em> 5’-ggctacaggtacctatgacccatctgga-3’</td>
</tr>
<tr>
<td>9</td>
<td>pRTdS-HA-YC</td>
<td>fw <em>(NheI)</em> 5’-ggaatcaggtacctatgacccatctgga-3’ and re <em>(XbaI)</em> 5’-ggctacaggtacctatgacccatctgga-3’</td>
</tr>
<tr>
<td>10</td>
<td>pRTdS-HsfA5-myc-YN</td>
<td>fw <em>(PagI)</em> 5’-gcctggaagcggacctata-3’ and re <em>(BglII)</em> 5’-ggctacaggtacctatgacccatctgga-3’ ligation into No. 10 cut <em>NcoI</em> and <em>NheI</em></td>
</tr>
<tr>
<td>11</td>
<td>pRTdS-HsfA5-HA-YC</td>
<td>triple ligation using same PCR fragments as for No. 12 into No. 11 cut <em>NcoI</em> and <em>NheI</em></td>
</tr>
<tr>
<td>12</td>
<td>pRTdS-HsfA4b-mYC-YN</td>
<td><em>NcoI</em>-NheI fragment from No. 1 cloned into No 10</td>
</tr>
<tr>
<td>13</td>
<td>pRTdS-HsfA4b-3HA</td>
<td>triple ligation using same PCR fragments as for No. 12 into No. 11 cut <em>NcoI</em> and <em>NheI</em></td>
</tr>
<tr>
<td>14</td>
<td>pRTdS-HsfA4b-mYC-YN</td>
<td><em>NcoI</em>-NheI fragment from No. 1 cloned into No 10</td>
</tr>
<tr>
<td>15</td>
<td>pRTdS-HsfA4b-3HA</td>
<td><em>NcoI</em>-NheI fragment from No. 1 cloned into No 11</td>
</tr>
<tr>
<td>16</td>
<td>pGST-HsfA4bCTD (112-393)</td>
<td>fw <em>(SalI)</em> 5’-ggctacaggtacctatgacccatctgga-3’ and re <em>(NotI)</em> 5’-gaattccctggaagcggacctata-3’</td>
</tr>
<tr>
<td>17</td>
<td>pGST-HsfA5CTD (110-478)</td>
<td>fw <em>(SalI)</em> 5’-ggctacaggtacctatgacccatctgga-3’ and re <em>(NotI)</em> 5’-gaattccctggaagcggacctata-3’</td>
</tr>
<tr>
<td>18</td>
<td>pJC-3HA-HsfA5-6His</td>
<td>fw <em>(XhoI)</em> 5’-ggctacaggtacctatgacccatctgga-3’ and re <em>(ApaI)</em> 5’-gaattccctggaagcggacctata-3’ PCR fragment cloned into pJC-HsfB1CTD-6His (22)</td>
</tr>
</tbody>
</table>
Fig. S1
Fig. S2

A

Signal intensities:

Seedling gr. Rosette leaves Stem Apex Flower Silique/Seeds Roots

Ubq11 HsfA4a HsfA4c HsfA5

B

shoot root culture

cold osmotic salt drought genotoxic oxidative UV-B wounding heat control cold osmotic salt drought genotoxic oxidative UV-B wounding heat

Ubq11 HsfA4a HsfA4c HsfA5

C

Set A Set B Set B

cold 1h MgCl2 1h MgCl2 1h DC3000 1h avrRpm1 1h HrcC 1h Paph 1h H2O 1h

cold 2h MgCl2 2h MgCl2 2h DC3000 2h avrRpm1 2h HrcC 2h Paph 2h H2O 2h

cold 6h MgCl2 6h MgCl2 6h DC3000 6h avrRpm1 6h HrcC 6h Paph 6h H2O 6h

cold 24h MgCl2 24h MgCl2 24h DC3000 24h avrRpm1 24h HrcC 24h Paph 24h H2O 24h

Ubq11 HsfA4a HsfA4c HsfA5
Role of heat stress transcription factor HsfA5 as specific repressor of HsfA4
Sanjeev Kumar Baniwal, Kwan Yu Chan, Klaus-Dieter Scharf and Lutz Nover

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