Arabidopsis Class A and B HSFs Show a Spectrum of Transcriptional Activity

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Summary

A plethora of heat shock transcription factors (HSFs) has been obtained from various plant species (33,45-48,50,51). The *Arabidopsis* genome sequencing project provided confirmation of the existence of at least twenty one HSFs which were classified into three major classes, A, B and C, and numerous subclasses (9). Members of HSF class A displayed differential transcriptional activities in tobacco protoplasts that varied from 15- to 50-fold above the control level. This diversity of activity levels may reflect HSF variations regarding their transcriptional activation functions- some of the members might be the major heat inducible HSFs (class A1 HSFs), while others act in an auxiliary capacity as HSF activity boosters (38). Two new class B HSFs showed no transcriptional activation potential. Reporter activities due to endogenous tobacco HSFs were inhibited by a class B HSFs showing high expression levels. This suppression of endogenous HSFs by class B members provides further evidence that class B HSFs are not transcriptional activators, but are able to *trans*-attenuate the transcriptional activity of *bona fide* activator HSFs (34,41). The transcriptional competency of class C HSFs has not been determined.

Key words:
transcription regulation, heat shock transcription factor, activation domain.

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1. Introduction

Heat shock transcription factors (HSFs) mediate a strong transcriptional response to heat shock (HS) and other stresses by a mechanism that is conserved in bacteria and higher eukaryotes, including plants (1). They bind to their respective DNA recognition sites in HS gene promoters (2), termed heat shock elements (HSEs; 3), and activate HS gene expression, an event that leads to the production of molecular chaperones protecting the cell from deleterious effects of stress. The HSE arrays of the 5 bp core sequence 5'-aGAAg-3', or its reverse complement 5'-cTTCt-3' (4), are present in various permutations in the proximal HS promoter juxtaposed to the TATAA box. HSEs can also reside in a distal promoter (5,6) where they seem to play a redundant role as documented for the soybean *Gmhsp17.5E* promoter (7).

2. Organization of HSF functional domains

2.1. DNA binding domain and a flexible linker

HSFs display highly conserved structural features (5,8,9). The N-terminally located DNA binding domain (DBD) of approximately 95 amino acids folds as a winged helix-turn-helix (H-T-H) and consists of a three-helix bundle and four antiparallel β-strands which form a β-sheet (10). Helix 3 may be responsible for making contacts with the first and second bases (a1 and G2) of the HSE core in the major groove of DNA (11). The wing was discovered to be a critical part of the mammalian HSF1 heat-stress-sensing mechanism (12). First, it confers DNA binding specificity and target gene preference of HSF family members (12), but it does not contact DNA itself (13). It operates through protein : protein interactions, possibly within a trimer or between adjacent trimers (13). Wing removal did not affect HSF protein stability nor ability to trimerize, but it decreased its DNA-binding affinity. This was seen in a deficiency of wingless HSF to form the first trimer-bound complex, but not the larger multimeric complexes. Wing-deletion experiments indicated that the wing was not involved in the highly cooperative nature of HSF binding, but rather it might stabilize the first trimer bound to DNA (13). Second, the wing suppressed formation of the HSF1 trimer under basal conditions and was needed for heat-inducible trimerization (12). Interestingly, plant HSF DBD does not seem to require the wing since the 11 amino acid loop located between β-strands 3 and 4 in other organisms is not present in tomato LpHSFB1 (16). Plant HSF DBDDBDs are separated from the oligomerization domains (OD) by a flexible linker (17) of 9 to 39 amino acid residues in class A, 50 to 78 in class B and 14 to 49 in class C HSFs (9).

In trimerized yeast (yHSF) that is not yet bound to the HS promoter, the flexible linker would permit a considerable movement of DBDs relative to one another. Such

swiveling might expose different surfaces of the DBD for contact with regulatory intramolecular regions or ancillary factors and would be eliminated by binding to HSEs (15). Intramolecular contacts between the DBD and other regions of HSF are evident in the case of yHSF where DBD directly interacts with the N-terminal transcriptional activation domain (AD), but not with the C-terminal one (15); yet, the DBD negatively regulates transcriptional activity of both the N-terminal and C-terminal ADs (14). One model of HSF regulation posits that the DBD senses heat directly through changes in its secondary structure, which results in increased ability to unmask the activation domains (14). The other (speculative) model of yHSF regulation suggests that the structurally constrained central domains, DBD and OD, may act to negatively regulate transcription and depress activity of transcriptional ADs through non-productive interactions with the elements of transcriptional machinery, basically competing with ADs for general transcription factors (GTFs). A HS induced conformational change of the central domains would eliminate non-productive complexes with GTFs, leaving transcriptional ADs to interact productively with the transcriptional machinery (15).

2.2. Nuclear localization signal

In the animal systems, one or two arrays of basic amino acids may be involved in directing the translocation of the HSF to the nucleus upon heat shock (18,19). The preferred nuclear localization signal (NLS) is located immediately adjacent to the C-terminus of the DBD, while the other resides downstream from the oligomerization domain. Recent studies indicate that the NLS plays a double role: not only is it a target of a nuclear transport receptor importin alpha, but in addition, it regulates oligomerization and DNA binding. These functions are separable within the NLS (20). In *Drosophila* HSF, deletion of the NLS resulted in a constitutive oligomerization and DNA binding (20), but in contrast, in avian HSF3 point mutations in the NLS inhibited trimer formation of cHSF3 (21). In tomato plants, only one K/R2 NLS signal (C-terminal to OD) is functional in directing HSF to the nucleus (22).

2.3. Negative region, trimerization domain and hydrophobic repeat HR-c

Two tiers of regulation apply in the case of animal HSFs. Human HSFs remain mostly in the cytoplasm as transcriptionally inactive monomers, or dimers, maintained in a dynamic heterocomplex with HSP90-immunophilin-p23 (23). Other synergistically interacting HSP chaperones, like HSP40 and HSP70/HSC70, are also implied in modulation of *Drosophila* HSF activity by a feedback repression (24). With the onset of stress signaled by the accumulation of non-native proteins, HSFs trimerize, translocate to the nucleus and bind to HSEs in HS promoters, but still remain transcriptionally inactive. The distinct negative regulatory domain (NR) located be-

tween the oligomerization domain and a C-terminal transcriptional activation domain is thought to repress the function of HSF activation domains AD1 and AD2 in a temperature-dependent manner (25-27). It is presumed to be a part of a heat-sensing mechanism that translates ultimately into a transcriptionally active HSF. No similar domain has been identified in plant HSFs. Acquisition of transcriptional competency seems to be correlated with hyperphosphorylation, as shown for human HSF1 (28,29), and it constitutes a second tier of regulation.

HSF multimerization requires the oligomerization domain that consists of two arrays of hydrophobic heptapeptide repeats, HR-a and HR-b (for review see 8,9,19, 30,31). Each amino acid residue has a designated location within this heptapeptide, $(4, 3\text{-}abcdefg)_n$, and *a* and *d* positions that are involved in the formation of a parallel triple stranded α-helical coiled-coil (32) are frequently occupied by leucine residues, hence the name "leucine zippers". Positions *e* and *g* are often occupied by charged amino acid residues and contribute to the stability of coiled-coil. In animal systems, HR-a/b regulates HSF homotrimer formation and directs HSF subcellular localization to the nucleus through intramolecular interactions with an analogous third, C-terminally located, hydrophobic repeat c (HR-c) (18).

In plants, there are three HSF classes: A, B and C (8,9,31,33-35). They differ significantly in their oligomerization domain and C-terminal regions from those of animal and yeast, and among each other. For class A HSFs, HR-a is shorter at its N-terminus, has an N-terminally extended HR-b and a unique insertion of 21 amino acids as compared with class B HSFs (31) where the reduction of HR-b length parallels the reduction/elimination of hydrophobic repeat HR-c. HR-c is known to cooperate with HR-b in masking the OD and keeping the HSF in an inactive monomeric state (18,36, 37). Since class B HSFs localize to the nucleus under control conditions (38), they are likely not to require this type of HS-induced regulation. One indication reflecting the diverged functional specialization of class A versus B HSFs may be the fact that tomato LpHSFB1 exists as dimer, while LpHSFA1 and A2 trimerize (9). The oligomerization domain of class C HSFs resembles that of class B; however, it seems to represent a bridge between both classes as it has an insertion of 7 amino acid residues joining HR-a and HR-b (9).

2.4. C-terminal regions/transcriptional activation domains

The C-terminal regions (CTRs) of plant HSFs are the least conserved in the aspect of sequence and size. They harbor transcriptional activation domains and in some cases HR-c and nuclear export sequences (NES). ADs were first elaborated for tomato HSFs as tryptophan-containing repeats (39) and subsequently remolded to AHA motifs (**a**romatic, large **h**ydrophobic and **a**cidic amino acids) (40). In class A representative AtHSFA4a, the AHA motif encompasses a consensus of two pairs of hydrophobic amino acids separated by two nonhydrophobic residues, *i.e.* FWqqFF

(AHA1) or IWenLV (AHA2) (41). Frequently, more than one AHA motif is required for transcriptional activity of an HSF. In tomato HSFs, AHA motifs are imbedded in an acidic region, contribute to activator potential and can substitute for each other with sequence and positional specificity (42). Similar types of transcriptional activation motifs were localized in activation domains of many mammalian HSFs and other transcription factors (40). Most importantly, AHA motifs are able to interact with TATAA-binding protein (TBP) and, as such, most likely recruit TFIID complex to the promoter nucleating the preinitiation complex (PIC) formation (31,43).

C-terminal regions of class B HSFs do not show any obvious conserved AHA or HR-c motifs. When tested *in vivo* in tobacco protoplasts in chimeric fusions with the DNA binding domain of yeast acidic activator Gal4, three class B HSFs from soybean and one from *Arabidopsis* were not transcriptionally active (41). Moreover, they were able to suppress transcriptional activities of 1) heterologous activation domain AD2 of human HSF1 when placed in *cis* in chimeric fusions, 2) endogenous tobacco HSFs 3) and co-expressed class A activator AtHSFA4a (41). Accordingly, class B HSFs may act as repressors of HS genes under basal conditions, and in principle, they have the potential to down regulate the HS response after the initial phase of induction (31,34). The supporting evidence for this stems also from the fact that HSFBs are localized to the nucleus under control and HS conditions (38).

The extreme C-terminal amino acids present in tomato LpHSFA2 have been shown to be involved in nuclear export (44). When fused with routinely nucleus-localized LpHSFB1, the nuclear export signal directed B HSF to the cytoplasm. The NES consensus motif is LnnnLnnLnL and has been compiled for known plant HSFs (for references see 9). It seems that class B HSFs do not have a strong NES.

Plant HSFs were first isolated from tomato (45) followed by *Arabidopsis* (46), maize (47) and soybean (33). The recent completion of the *Arabidopsis* genome project resulted in the discovery of many more HSFs. Attempts were made to classify them into various groups based on their sequence homology, structural similarities and functional aspects (9,31,35). Current indications suggest that among identified 21 HSFs there are at least three multimember classes designated A, B and C. The plethora of HSFs are believed to have arose through exon shuffling and subsequent elimination of all but one (highly conserved as to the localization within the DBD) ancestral intron resulting in the generation of HSFs of mosaic construction (9).

3. Materials and methods

3.1. RT-PCR and cloning of selected *Arabidopsis* HSFs

Several *Arabidopsis* HSFs identified previously, or newly introduced to the world database by the *Arabidopsis* genome sequencing project, were cloned using RT-PCR (Ta-

ble 1). The rosette leaves of *Arabidopsis* var. Columbia were ground in a mortar with pestle using RNAwiz Isolation Reagent from Ambion (cat. No 9736) at a ratio of 1 ml per 100 mg of tissue. Total RNA was isolated according to the manufacturer's instructions and applied as a template in RT-PCR reactions performed with ProStar Ultra High Fidelity kit (cat. No 600166) from Stratagene using either oligo(dT)$_{18\text{-}20}$ or HSF-specific downstream primer for the first strand cDNA synthesis. Subsequently, open reading frames of individual HSFs were amplified from the first methionine to the last amino acid for each HSF. The primers had engineered restriction sites for further cloning. These were Sal I upstream from the start codon, and Bgl II or Not I downstream from the last amino acid. Primers were designed with the use of the Oligo4.0 computer program. HSF amplicons were separated from unreacted primers with the Concert Rapid PCR Purification System from GibcoBRL (cat. No 11458), digested overnight with restriction enzymes Sal I/Bgl II or Sal I/Not I, resolved on agarose gels away from cleaved ends and recovered from gels with the Concert Rapid Gel Extraction System from GibcoBRL (cat. No 11456). HSF cDNAs were then ligated into a modified pBI221-based vector from Clontech Laboratories, Inc. for transient *in vivo* expression in tobacco protoplasts. The modification involved removal of β-glucuronidase (GUS) coding sequence and insertion of Gal4 leader sequence as previously described (41).

Table 1

HSFS from *Arabidopsis thaliana* expressed in tobacco protoplasts

Name	Protein Accession	Class
AtHSFA1a (AtHSFA1-1)	CAA53761	A1
AtHSFA1b (AtHSFA1-3)	CAA74397	A1
AtHSFA1e (AtHSF2)	AAF26960 CAB63800	A1
AtHSFA4a **AtHSFA4a*** (AtHSFA4-21)	CAA16745 (genomic) AAC31792 (cDNA*)	A4
AtHSFA5/A4b (AtHSFA4-2244754)	CAB10177	A5/A4
AtHSFA7a (AtHSFA2-4678920)	CAB41311	A7
AtHSFB1 (AtHSFB1-4)	U68017[1] (cDNA) CAB16764 (genomic)	B1
AtHSFB2b (AtHSFB-29)	CAB39937	B2
AtHSFB3 (AtHSFB-2618703)	AAB84350	B3

In parentheses; HSF previously classified name (31,35). In bold: currently assigned name (9). * = nucleotide data base accession number.

Tobacco protoplast isolation, harvesting, GUS and luciferase (Luc) assays were conducted according to established protocols (41).

4. Results and discussion

4.1. Relations of tested HSFs

Recent information from the *Arabidopsis* genome sequencing project made it quite evident that plants differ significantly from other systems in the number of their HSF genes. While *Drosophila*, yeast, nematode *C. elegans* and frog *X. laevis* have one HSF, and birds and mammals three, plants seem to have expanded their need for HSFs. Tomato, maize, and soybean express at least three to six HSFs (33,45,47), while in *Arabidopsis* twenty one HSFs have been classified (9). We have used RT-PCR for cloning of these HSFs (Table 1) to study their transcriptional activation potential in tobacco protoplasts (Fig. 1).

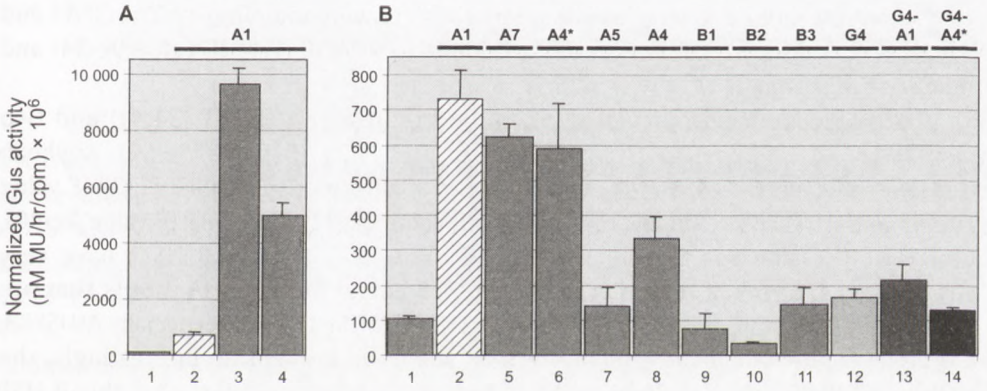

Fig. 1. Transcriptional activity of *Arabidopsis* HSF members from A and B classes. The effectiveness of full length HSFs in transcriptional activation was tested *in vivo* in tobacco mesophyll protoplasts as a reflection of GUS reporter activity driven by HSEs upstream from the TATA box. GUS activity was normalized to the activity of the luciferase gene driven by the ubiquitin promoter (Y-axis). The yeast Gal4 leader construct (lanes A1 and B1) represents transcriptional activity of endogenous tobacco HSFs binding to HSEs in the GUS reporter promoter. As a point of reference and a positive control, tomato LpHSFA1 (39) was included as shown in lanes A2 and B2 (stripped bar). The following *Arabidopsis* HSF effectors were over-expressed from the A1 class: AtHSFA1b (lane 3), AtHSFA1e (lane 4); from the A7 class: AtHSFA7a (lane 5); from the A4 class: AtHSFA4a* (lane 6), AtHSFA5/A4b (lane 7) and AtHSFA4a (lane 8). Class B representatives include AtHSFB1a in lane 9, AtHSFB2b in lane 10 and AtHSFB3 in lane 11. The activity of chimeric AtHSFA1a and AtHSFA4a* fused to yeast Gal4DBD147, and Gal4DBD147 alone, are shown in lanes 13, 14 and 12, respectively. Asterisk denotes N-terminally mutated AtHSF4a clone (see text).

Previously, from an *Arabidopsis* cDNA library we obtained an activator AtHSFA4a (AAC31792) represented by a partial cDNA. At the N-terminus it contained an Eco R I site, and we engineered the first methionine codon in frame upstream. Comparison with genomic sequence of AtHSF4a (CAA16745) indicated that the Eco RI site was an artifact of the cDNA library preparation. Hence, AtHSFA4a and AtHSF4a* constructs differ at their extreme N-terminus by 4 amino acid residues; MDE is present in the genomic and MEFR in the cDNA.

AtHSFA1e is a putative HSF closely related to AtHSFA1b (48). It shows all the characteristic structural features of plant HSF, including two putative AHA motifs FWeqFI and VWskNQ in its C-terminal region. However, information regarding its transcriptional activity has not been reported. Another new HSF is AtHSFA7a that shows homology to AtHSFA7b (E value from BLAST search was 5e-48), AtHSFA6b/a pair (E values 4e-39 and 5e-36, respectively) and tomato LpHSFA1 (E value 6e-36). Heat shock transcription factor-like protein AtHSFA5/A4b has a second open reading frame connected to its N-terminus, which results in a total of 834 amino acid residues. In generation of the AtHSFA5/A4b construct, the methionine 369 (20 amino acids upstream from HSF DBD) was used as a start codon giving an open reading frame (ORF) of 466 amino acids. A BLAST search with ORF466 indicated that AtHSFA5/A4b was identical to a newly submitted *Arabidopsis* sequence NP_567415 and it showed significant similarity with the putative HSF from *Oryza sativa* (BAB56047; E value 1e-35) that has not yet been classified, but itself showed homology to ZmHSFA4 and NtHSFA4. In addition, ORF466 showed homology to AtHSFA1a (E value 9e-34) and AtHSFA1e (E value 1e-33).

Class B HSFs include previously identified repressor AtHSFB1 (34,41) and two new HSFs. AtHSFB2b shows homology to AtHSFB2a (E value 5e-55), soybean GmHSFB4a (E value 7e-45) and soybean GmHSFB3a (formerly GmHSF29 (33)) (E value 1e-44); and AtHSFB3 is similar to NtHSFB1 (E value 1e-42), LpHSFB1 (E value 2e-40), GmHSFB1 (E value 9e-38) and AtHSFB1 (E value 1e-25). Class B HSFs have very streamlined C-terminal regions. They do not seem to harbor AHA motifs that are prerequisites for transcriptional activation. CTRs of AtHSFB2b and, especially AtHSFB3, are highly acidic while the CTR of AtHSFB1 seems to be neutral. Interestingly, the CTR of AtHSFB3 might include a NES, which opens the possibility that this B HSF may be able to translocate out of the nucleus. From studies done in tomato, it is known that Class B1 HSFs routinely localize to the nucleus and do not have an obvious NES (9,38).

4.2. Differential transcriptional activation potential of class A and B members

Analysis *in vivo* in tobacco protoplasts of class A and B HSF members clearly indicated that there were significant differences in transcriptional competency between subclasses (Fig. 1). Among tested HSFs, AtHSFA1b and AtHSFA1e displayed the high-

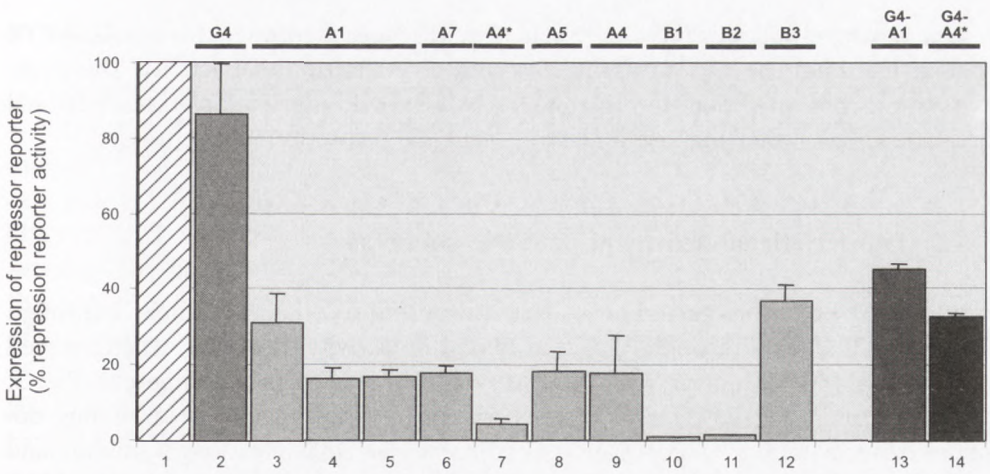

Fig. 2. Expression of functional proteins assessed by DNA binding. The activity of the repressor reporter in the presence of over-expressed Gal4 leader construct and endogenous tobacco HSFs constitutes 100% activity and is shown in lane 1 (checkered). Gal4DBD147 replaced the Gal4 leader construct in lane 2. The effectors of class A HSFs were as follows: tomato LpHSFA1 (lane3), AtHSFA1b (lane 4), AtHSFA1e (lane 5), AtHSFA7a (lane 6), AtHSFA4a* (lane 7), AtHSFA5/A4b (lane 8) and AtHSFA4a (lane 9). Class B HSFs: AtHSFB1a, AtHSFB2b and AtHSFB3 are shown in lanes 10, 11 and 12, respectively. Two chimeric HSF constructs containing the Gal4DBD147 fused to the N-terminus of *Arabidopsis* HSFs are presented in lane 13 for AtHSFA1a, and lane 14 for AtHSFA4a*.

est transcriptional activity (Fig. 1A lanes 3 and 4). Tomato LpHSFA1 was 10-fold less active than *Arabidopsis* HSFA1b (lane 2A). Its activity was comparable to activities of AtHSFA7a and AtHSFA4a* (Fig. 1B lanes 2, 5 and 6, respectively). It is noteworthy that when isolated transcriptional activation domains of these three class A1 HSFs were tested as Gal4 fusions, activity levels were comparable to AtHSFA1a (43). These unexpected high activities for the CTRs may be indicative of an unidentified additional regulatory element that suppresses the activity of the CTR in a the context of the full length tomato protein.

There was an approximately 40% difference in transcriptional activity of AtHSFA4a* and A4a constructs (Fig. 1B lanes 6 and 8). We attribute this difference to changed amino acid sequence at the N-terminus of the cDNA construct. It is possible that mutation in such close proximity to the HSF DBD might affect the folding properties of the DBD and influence its ability to bind to HSEs (see Fig. 2), or alternatively it might affect HSF expression through changes in protein stability. However, this mutation does not affect the CTR's activity directly; transcriptional activities of chimeric Gal4 fusions of isolated CTRs for both A4s were similar (data not shown). The activity of AtHSFA5/A4b was minimal and questionable, but low activities were further confirmed by Gal4/CTR fusion studies (43).

As predicted, neither full length (FL) (Fig. 1B, lanes 9 through 11) nor Gal4/CTR fusions (data not shown) of class B HSFs showed transcriptional activity. This demonstration confirmed that, in principle, class B HSFs do not work as transcriptional activators, but rather may be a class of *bona fide* transcriptional repressors.

4.3. Transcriptional activity of Gal4/HSF chimeras

Gal4/HSF FL fusions tested on an HSE-driven reporter (Fig. 1B, lanes 12 through 14 or Fig. 3, lanes 3 through 5) also exhibited no activity. However, when assayed on a 10xGal4/GUS reporter, Gal4 fusions were transcriptionally competent (Fig. 3, lanes 6 through 8). Gal4/HSF FL fusions contained both types of DNA binding domains: N-terminal Gal4DBD147 and the adjacent HSF DBD (Fig. 3 right, middle and bottom panels). The mode of the HSF chimeras' binding to the respective DNA binding sites present in two GUS reporters remains a subject for speculation. Although the native HSFs seem to predominantly trimerize and, as such, bind to HSEs (Fig. 3 right, top), the chimeras may bind to their respective DNA binding sites in a variety of structural combinations (Fig. 3 right, middle and bottom) which in some cases may be linked to the loss of transcriptional activity (Fig. 3 right middle, stop sign).

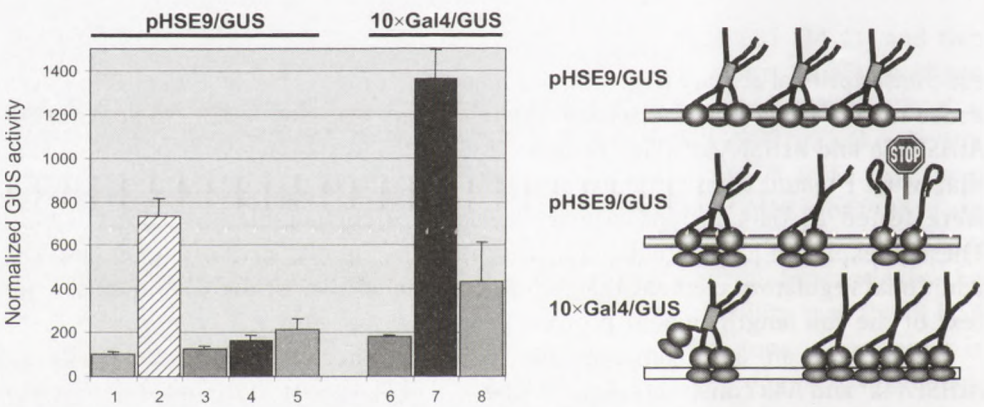

Fig. 3. Gal4DBD fusions to N-termini of *Arabidopsis* HSFs inhibit their transcriptional activity. The endogenous activity of tobacco HSFs activating HSE-driven GUS reporter in tobacco protoplasts are shown in the presence of over-expressed Gal4 leader construct (lane 1) or Gal4DBD147 (lane 3), while N-terminal Gal4DBD-HSF fusions are present for AtHSFA1a (lane 4) and AtHSFA4a* (lane 5). Native tomato LpHSFA1 served as a positive control (lane 2). These same two *Arabidopsis* HSFs tested on the GUS reporter driven by 10 copies of Gal4 DNA binding sites upstream from TATA are depicted in lanes 7 and 8, respectively, with Gal4DBD147 alone in lane 6. The diagrams on the right indicate possible binding configurations of native (top) or Gal4DBD-chimeric (bottom two) HSFs to HSE or Gal4 DNA binding sites. Round HSF native DBD; oval = Gal4DBD; rectangle trimerization domain.

4.4. HSF protein expression levels tested by suppression of GUS repressor reporter

To ascertain that the lack of transcriptional activity of HSFs was not caused by an inability to express HSF protein in tobacco protoplasts, we quantified DNA-binding activities of expressed proteins using a GUS repressor reporter as described earlier (39,41). The repressor reporter driven by the 35S CaMV promoter has 9 perfect consensus HSE sites introduced into the leader sequence. Expressed effector proteins that contained HSF DBDs bind to HSEs within the leader and repress the expression of GUS. The level of this repression was assumed to proportionately reflect the level of HSF protein expression. This functional method of assaying HSF DBD binding to HSEs substituted for direct protein detection by Western blot analysis that was hampered by the lack of adequately sensitive anti-HSF antibodies. Additionally, it was assumed *a priori* that the differential DNA binding affinities of individual HSF DBDs would be minimized on a DNA recognition site that consisted of an array of perfect HSEs.

Arabidopsis HSFs listed in Table 1 were tested for their effectiveness in repression of GUS repressor reporter activity (Fig. 2). As expected, the Gal 4 DBD had a minimal effect on the repressor reporter when expressed alone (compare lanes 1 and 2), while all HSF constructs repressed GUS activity (lanes 3 through 14). AtHSFA1b, A1e, A7a (lanes 4 through 6), A5/A4b and A4a (lanes 8 and 9) seem to be expressed well and at the same level since they displayed approximately 80% of GUS repression. Therefore, the differences in their transcriptional activities presented in Fig. 1 seem to reflect the intrinsic transcriptional potential of each individual HSF (lanes 3, 4, 5, 7 and 8, respectively). The increased transcriptional activity of AtHSFA4a* construct (N-terminal mutant) may be partially explained by its more efficient binding to HSEs or by a more stable protein as compared to the wild type construct (Fig. 2, lanes 7 and 9). Conversely, the somewhat low activity of tomato LpHSFA1 (Fig. 1A and B, lanes 2) might stem from a lower level of protein expression (Fig. 2, lane 3). Two out of three class B HSFs, AtHSFB1 and B2 were extremely well expressed and repressed the activity of the repressor reporter 99 and 98%, respectively (lanes 10 and 11). In contrast, AtHSFB3 inhibited GUS expression only 60% (lane 12), which was also the approximate level of repression displayed by two Gal4DBD/HSF chimeric constructs (lanes 13 and 14). The lack of activities for AtHSFB3 and Gal4DBD/HSF chimeras was, therefore, not the result of poor protein expression since all three constructs clearly bound HSEs and repressed GUS activity. In the case of Gal4DBD/HSF chimeras, the addition of Gal4DBD at the N-terminus of HSFs must inhibit the transcriptional activity of the HSFs by undetermined mechanism. This interpretation is consistent with studies in yeast where there is precedence for DNA binding domain inhibition of activation domains (14).

5. Conclusions

Arabidopsis HSFs, identified by the genome project and considered to be either putative (AtHSFA1e, AtHSFA7a, AtHSFB3) or HSF-like (AtHSFA5/A4b, AtHSFB2b), are indeed present in the rosette leaf mRNA population and seem to be ubiquitous. When expressed *in vivo* in tobacco protoplasts, they were functionally competent either as activators- AtHSFA1a, A1b, A1e, A4a, A5/A4b, A7a- or as strong repressors of endogenous tobacco HSFs- AtHSFB1 and AtHSFB2b. AtHSFB3, albeit expressed as a protein, exhibited neither activator nor repressor-type activity. This HSF is the smallest of the *Arabidopsis* HSFs with an ORF of only 244 amino acids and CTR consisting of approximately 26 amino acid residues. This terminal region shows homology to the NES consensus, and is very acidic with 13 amino acid residues being negatively charged. So far, the function of this HSF remains unknown; however, it is feasible that it may not be involved in the attenuation of the heat shock response as postulated for most of class B HSFs, but, perhaps, has specialized for some alternate role. Another example of a diverged functional role may be exemplified by MsHSFA4a which seems to be involved in the cold acclimation of alfalfa plants (49), a function clearly outside traditional expectations.

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Abbreviations

Abbreviations used in current text have been shown alphabetically:
AD – transcriptional activation domain
CaMV – cauliflower mosaic virus
DBD – DNA binding domain
GUS – β-glucuronidase
HR – hydrophobic heptapeptide repeats a-, b- and c
HS – heat shock
HSF – heat shock transcription factor
HSP – heat shock protein
NES – nuclear export signal
NLS – nuclear localization signal
NR – negative regulatory domain
OD – oligomerization domain

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