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Arabidopsis heat shock factor: isolation and characterization of the gene and the recombinant protein [+)]

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Abstract

We have isolated the *Arabidopsis* heat shock factor gene *Athsf1* as genomic and corresponding cDNA sequences via cross-hybridization with tomato clones. Sequence analysis indicates only a partial homology with the HSFs from tomato and other organisms which is confined to the DNA-binding and the oligomerization domains. The gene is constitutively expressed but the level of mRNA for *Athsf1* increases two-fold upon heat shock. However, the putative promoter region lacks the canonical heat shock elements. After expression in *Escherichia coli* the recombinant Athsf1 protein binds specifically to a synthetic oligonucleotide containing five heat shock elements. The native size of recombinant ATHSF1 *in vitro* is consistent with a trimer as demonstrated by chemical cross-linking and pore exclusion limit analysis.

Introduction

The heat shock (hs) response is a conserved reaction of cells and organisms responding to environmental stress, particularly to elevated temperatures, and in different organisms also to a variety of chemical inducers. A common feature of the hs response is the rapidly induced expression of heat shock proteins (Hsp). For most of the different classes of Hsp (Hsp70, Hsp60 and Hsp20) a functional role as molecular chaperones has been demonstrated (see [15, 21] for review). In plants Hsp20 seems to play a special role because this class of Hsp is prevalent and members of the Hsp20 family are also transported into chloroplasts. Their expression is primarily regulated at the transcriptional level; post-transcriptional regulation such as control of translation and mRNA stability are secondary phenomena.

In eukaryotes the induction of the transcription of heat shock genes is mediated by a pre-existing transcriptional activator, the heat shock factor (HSF), which binds to a conserved sequence, the heat shock element (HSE), present in multiple copies in the promoter regions. The binding motif of HSE is composed of 5 bp [nGAAn] blocks in alternating orientations; at least three units are required for stable binding [2, 23, 33, 38, 40]. In *Drosophila*, vertebrates and plants binding of HSF to HSE is hs-inducible [18, 28, 29] and requires

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X76167.

[+)] The publication is dedicated to Professor Dr. Wolfram Heumann, Universität Erlangen, on occassion of his 80th birthday.

in vertebrates, *Drosophila* and yeast a conversion from a latent monomer to an active trimer [6, 20]. Hence HSF is a constitutively expressed gene whose function is post-transcriptionally controlled.

Genes encoding HSF have been isolated from the yeasts *Saccharomyces cerevisiae*, *K. lactis* and from fruit fly, chicken, mouse, man and tomato (see [20] for review). In yeasts and *Drosophila* HSF is encoded by a single gene shown to be essential for yeast [35]. Somehow unexpected has been the multiplicity of HSF, in tomato by three [29] and in several other species including man [30], mouse [28] and chicken [22] by at least two different genes. The precise functional role of different HSF is not known but transient expression of tomato HSF in tobacco suggests differences in promoter specificity [36].

Sequence comparisons of HSF genes from different species demonstrate a strong conservation but only in their DNA-binding and oligomerization domains [28]. This holds true also for the three tomato HSF genes which have been isolated via screening of cDNA libraries using synthetic HSE as DNA ligand [29].

Here we describe the identification of a cDNA and the corresponding genomic sequences from *Arabidopsis thaliana* that show the characteristic motifs of HSF. The recombinant protein binds to synthetic HSE sequences and forms a multimer *in vitro*. The expression and function of the *Arabidopsis* HSF is discussed. The identification of this regulatory gene in *Arabidopsis* and the characterization of the recombinant protein are essential for the genetical, molecular and biochemical analyses of the heat stress-induced signal transduction in this plant model.

Material and methods

Library screening

An *Arabidopsis* genomic library in λDash and a cDNA library in λgt10 derived from adult, non-heat-shocked plants were used. The genomic library was screened by hybridization with tomato cDNA clones Lphsf8 and Lphsf24; the cDNA library with Lphsf8, Lphsf24 in addition to the crosshybridizing fragments of the genomic screen.

Hybridization was performed at 38 °C in 10 mM PIPES pH 6.9, 600 mM NaCl, 1 mM EDTA, 10 × Denhardt's solution, 0.1% SDS, and 100 μg/ml yeast RNA. The final wash was done in 0.2 × SSC at 50 °C. Six genomic and four cDNA clones were isolated, subcloned into pBluescript SK (Stratagene) and sequenced.

Sequence analysis

Deletion derivatives were generated using exonuclease III [22] and cloned into pBluescript. Nucleotide sequences were determined by the dideoxynucleotide method [26]. Both strands of the sequence presented in Fig. 1 were determined.

All cloning and DNA manipulations including also primer extention mapping of the transcriptional start site were carried out according to standard procedures [25].

Heat shock conditions and nucleic acid isolation

A hs treatment of 37 °C or the control temperature (25 °C) was applied for 2 h to whole *Arabidopsis* plants as described for soybean seedlings [16].

Total RNA was isolated exactly as described [4]. For preparation of genomic DNA the method of Dellaporta *et al.* [8] was applied.

Electrophoresis of DNA and RNA and hybridization was carried out as described [31].

Reprobing the northern blot with 18S and 25S rDNA proved that equal amounts of RNA were present in each lane.

The mRNA levels were quantified by radioisotope scanning (Berthold).

Cloning and expression in E. coli

For the expression of the *Athsf1* protein the QIA-express expression system (Diagen, QE1003) was used.

The cDNA was cloned *Sal* I/*Pst* I into the pQE-32 expression vector. In this construct a 6 × His tag is located 5′ to the polylinker. Expression and purification was done according to the manufacturer's description.

Gel retardation assay

1 μg poly[dIdC] and 1 ng ^{32}P-labelled HSE oligonucleotide in 25 μl binding buffer (20 mM Tris-HCl pH 8, 50 mM NaCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 10% glycerol) were incubated with 1 μg recombinant, purified ATHSF1 protein. After 25 min at 25 °C the mixture was electrophoresed overnight on a 5% polyacrylamide gel (acrylamide/bisacrylamide 47:1) with TBE as running buffer. The following oligonucleotides were used for the gel retardation assay:

HSE 5′-tcgaggatcctaGAAgcTTCcaGAAgcTTCtaGAAgcagatc
cctaggatCTTcgAAGgtCTTcgAAGatCTTcgtctagagct-3′

HSEm 5′-tcgaggatcctatAAgcTTacatAAgcTTatatAAgcagatc
cctaggataTTcgAAtgtaTTcgAAtataTTcgtctagagct-3′

Oligonucleotides were annealed at 25 °C overnight, the double strand eluted from a 8% polyacrylamide gel and labelled with ^{32}P-ATP using T4 kinase.

Pore exclusion limit gel electrophoresis [3]

5 μg of recombinant, purified ATHSF1 protein and high molecular weight marker proteins (Pharmacia Lot 3070445011) were electrophoresed on a 3–15% polyacrylamide (2–10% glycerol) gradient gel in 0.5 × TBE buffer. Electrophoresis was continued for 24 h at 4 °C (20 V/cm) and the gel was stained with Coomassie blue.

Chemical cross-linking [1]

Cloned recombinant HSF (5 μg) was incubated with ethyleneglycol-bis(succinic acid *N*-hydroxysuccinimide ester) (EGS from Sigma), dissolved in dimethyl sulfoxide, for 30 min in 10% v/v reaction buffer (150 mM NaCl, 25 mM sodium phosphate, 10% glycerol). Reactions were quenched by the addition of 10% v/v of 300 mM glycine, precipitated with 15% TCA, washed twice with ice-cold acetone and dissolved in Laemmli sample buffer. Samples were heated to 95 °C for 5 min and separated on an SDS/4–10% polyacrylamide gel without a stacking gel and stained with Coomassie blue.

Results

Isolation of Arabidopsis *HSF genes*

The screening of libraries, cDNA in λgt10 and genomic DNA in λDash, was based on cross-hybridization of the DNA-binding domain with tomato cDNA clones for hs transcription factors Lphsf8 and Lphsf24. The genomic screen resulted in several independent clones which were subdivided in three groups based on restriction mapping and DNA sequence analysis (data not shown). The cross-hybridizing fragments and the tomato genes were used as probes for cDNA library screening. Four out of 500 000 clones were identified and characterized by DNA sequencing. All of them shared identical sequences corresponding to only one of the genomic sequences. The longest cDNA clone is obviously not full-length, missing the 5′ terminus of the protein-coding sequence. Comparative analysis of cDNA and genomic sequences revealed that the longest possible open reading frame of 1473 nucleotides is interrupted by an intron of 148 nucleotides located within the DNA-binding domain.

The open reading frame encodes a protein of 491 amino acids with an estimated molecular mass of 54 kDa.

The transcriptional start site was determined by primer extension (data not shown); it is located ca. 374 nt upstream from the protein coding sequence (Fig. 1). The promoter region upstream from the transcriptional start site contains two CCAAT boxes (positions −421 and −158); it

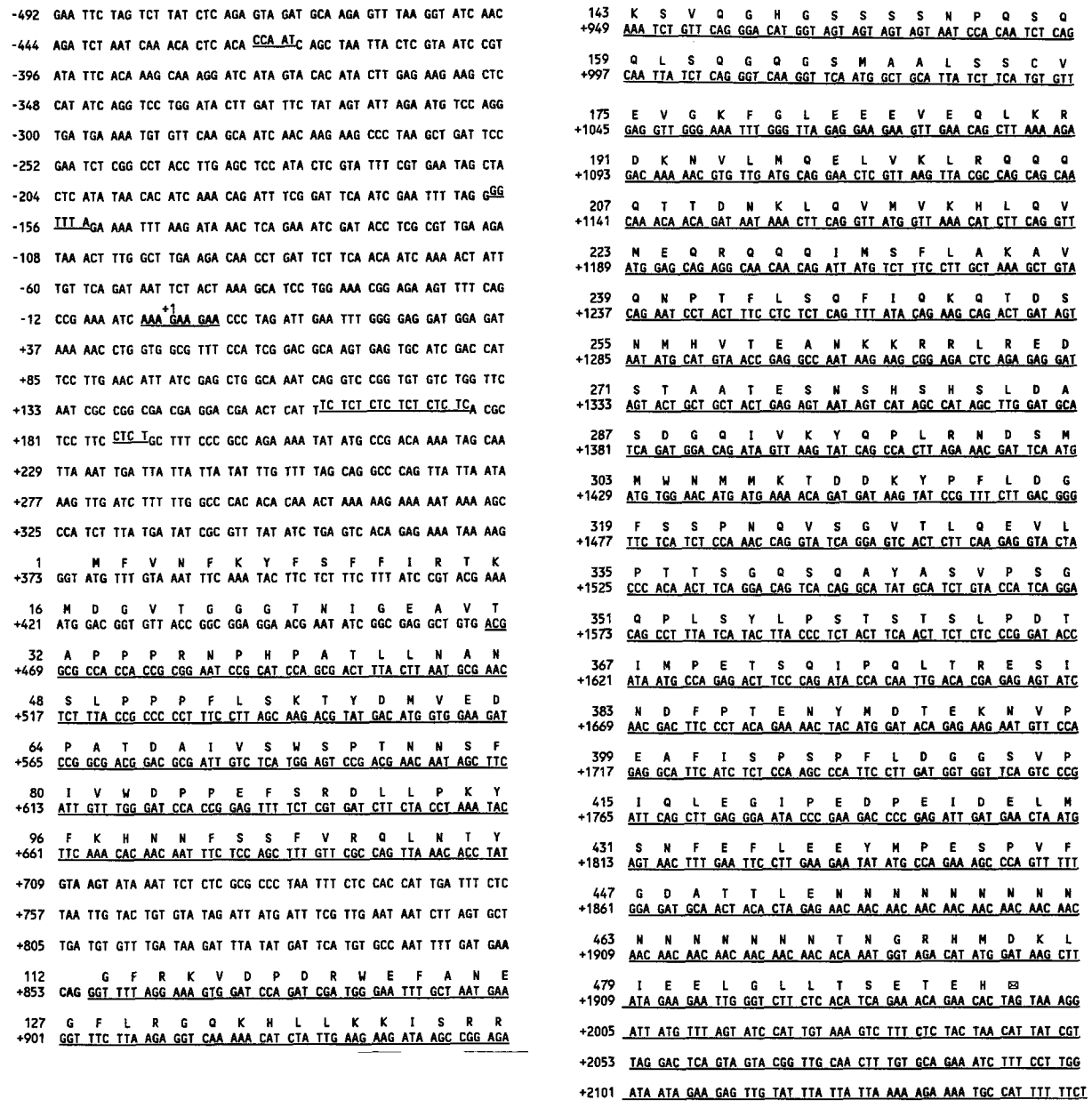

Fig. 1. Nucleotide and predicted amino acid sequences. The sequence was obtained by sequencing a set of overlapping exonuclease III deletions of the cDNA and genomic clones. The cDNA sequence is underlined, intron borders (nt 1201 and nt 1348) are highlighted. The transcription start site determined by primer extension (nt + 1), the CCAAT boxes (nt − 421, −158) and the GA stretches (nt + 161, + 187) are indicated by upper-case characters.

lacks sequences that resemble the classical TATAAA motif. Downstream from the transcriptional start site at position + 161, a 16 bp region of alternating G and A residues was found (Fig. 1). Such repeats have been shown to be essential for DNaseI hypersensitive site formation within the *Drosophila Hsp26* gene [19].

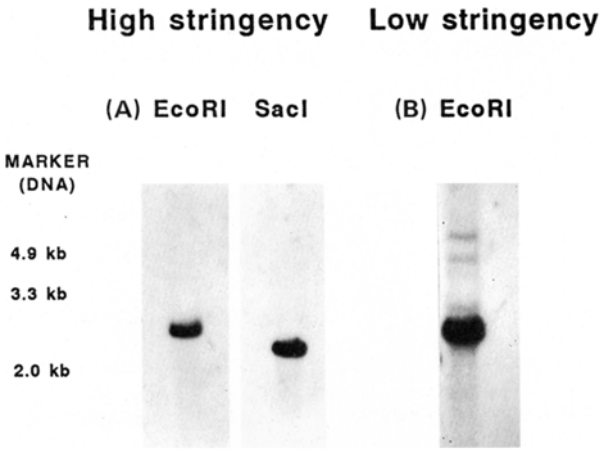

Fig. 2. Southern blot analysis of *Athsf1*. Genomic DNA (10 μg/lane) was digested with the restriction enzymes as indicated, separated by agarose gel electrophorese, blotted onto a nylon membrane and probed with an *Athsf1* genomic fragment (nt −492 to +1822). A. The blots were hybridized at 42 °C and washed to final conditions of 65 °C, 0.2 × SSC, 0.1% SDS. B. The blot was hybridized at 30 °C and washed to final conditions of 35 °C, 0.2 × SSC, 0.1% SDS. Migration of DNA size markers is indicated at the left.

Athsf1 *is constitutively transcribed*

Southern blot analysis of *Arabidopsis* genomic DNA digested with *Eco* RI or *Sac* I and probed with an *Athsf1* genomic fragment (nt positions −492 to +1822), which includes promoter and protein-coding regions, suggest that *Athsf1* is present as a single-copy gene (Fig. 2A). A tandem duplication of the gene is very unlikely since further sequencing extending in the Athsf flanking regions failed to identify any duplications (F. Schöffl, unpublished results). Hybridizations carried out under low stringency conditions show at least two supplementary cross-hybridizing fragments (Fig. 2B), indicating the presence of related but not identical genomic sequences in the *Arabidopsis* genome.

Northern blot hybridization of RNAs from control and heat-shocked *Arabidopsis* plants with a gene-specific 41 bp oligonucleotide probe derived from the untranslated leader sequence (+53 to +93, downstream of the transcription start site) shows only a single hybridizing band (Fig. 3). A rehybridization of the same blot using the whole cDNA enhanced strongly the hybridization signal but no additional cross-hybridizing bands appeared (Fig. 3). The size of the *Athsf1* mRNA is ca. 2100 nucleotides, which is in good agreement with the size of the transcript deduced from the sequencing data (2011 nt plus poly(A) tail). The

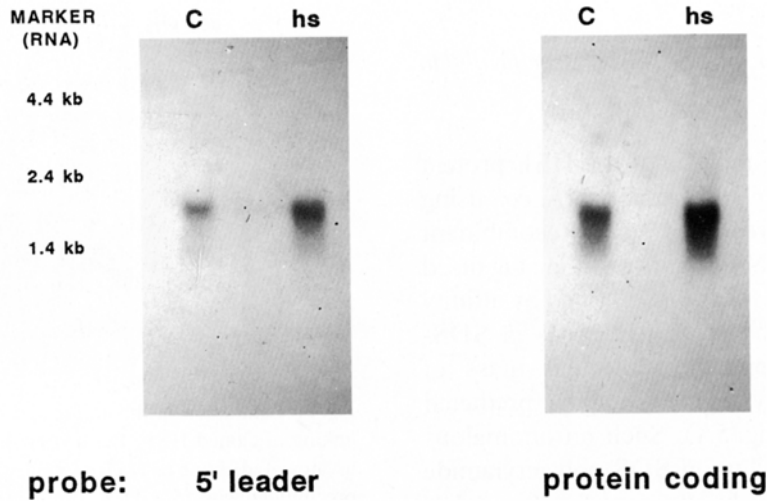

Fig. 3. Northern blot analysis of *Athsf1* in *Arabidopsis thaliana*. 40 μg RNA from control (c) and heat shocked (hs) plants were separated by agarose gel electrophoresis, blotted on nylon and probed (left) with an oligonucleotide (nt +53 to +93) derived from the sequence of the 5′ leader and (right) rehybridized with *Athsf1* cDNA (nt +466 to +2077). Hybridizations were carried out at 60 °C and washed to final conditions of 60 °C, 0.2 × SSC, 0.1% SDS. Migration of RNA size markers is indicated at the left.

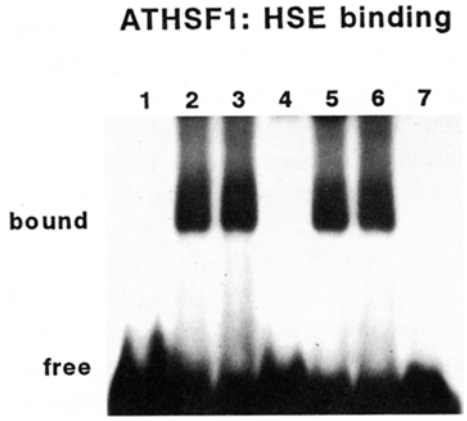

Fig. 4. Gel shift analysis. For the gel retardation assay a double-stranded 42 nt oligonucleotide containing five perfect HSE was used. Complex formation with the labelled HSE (lanes 2, 3) is efficiently competed by a 100-fold excess of the unlabelled HSE (lane 4). The DNA-protein complex is not competed by a 100-fold excess of a mutant oligonucleotide HSEm (lane 5, 6). No complex is formed with the labelled HSEm (lane 7). Negative control (lane 1): instead of ATHSF1 protein BSA was incubated with the labelled HSE oligonucleotide.

differences in *Athsf1* mRNA levels between hs and control tissues were quantified by radio isotope scanning and showed roughly a two-fold increase upon heat shock (data not shown).

Recombinant Athsf1 *protein binds specifically to HSE*

For *in vitro* binding analysis of the HSF protein *Athsf1* cDNA was overexpressed in *E. coli* using the QIAexpress expression system. Recombinant HSF containing an N-terminal histidine tag fused to amino acid 31 of *Athsf1* was purified by affinity chromatography. The protein migrates in SDS-PAGE with an apparent molecular mass of 80 kDa, significantly higher than the predicted value of 54 kDa (Fig. 5A). Such an anomalous electrophoretic mobility on SDS-polyacrylamide gel has been previously observed for *Drosophila* and human HSF proteins [5]. The DNA-binding ability of the recombinant *Athsf1* was shown by gel mobility shift using synthetic HSE sequences (Fig. 4, lanes 2, 3). Specificity of the HSF/HSE

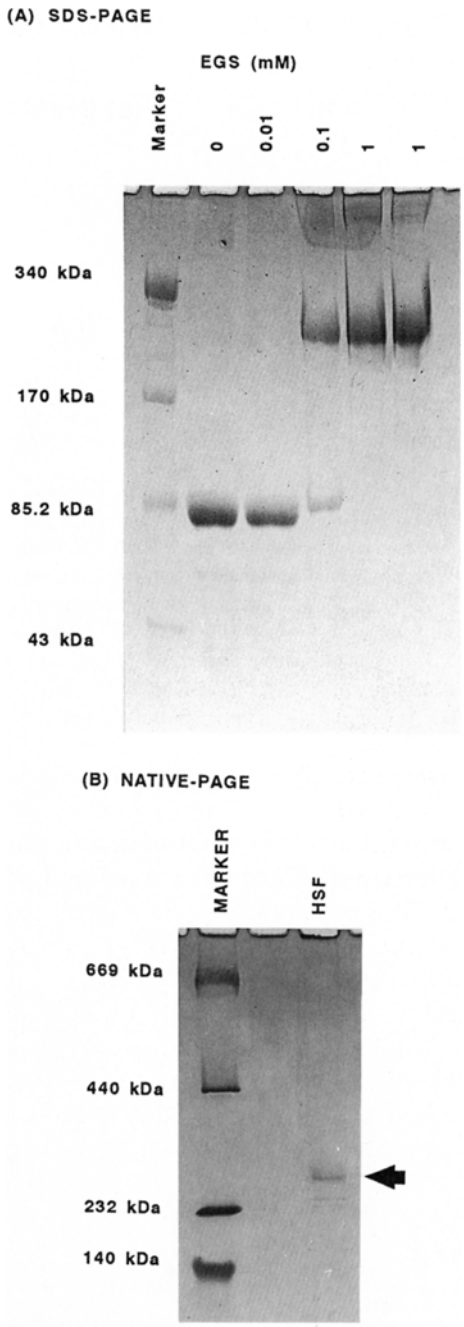

Fig. 5. Estimation of the nature size of HSF. A. EGS cross-linking of cloned HSF. Purified HSF was treated with EGS as indicated. Lane 6: HSF protein was diluted 100-fold and precipitated with 15% TCA. Proteins were separated by SDS-PAGE. B. Pore exclusion limit analysis of cloned HSF. 5 μg cloned HSF was electrophoresed on a nondenaturating 3–15% polyacrylamide gel until the limit of migration was reached. The gels were stained with Coomassie blue. Positions of marker proteins are indicated.

complex was determined by a competition using 100-fold excess of unlabelled HSE oligonucleotide (lane 4). A mutated oligonucleotide was unable to compete the labelled DNA fragment to that complex formation was not affected (lanes 5, 6).

Oligomeric state of recombinant HSF

The native size of recombinant HSF was measured by pore exclusion limit analysis according to Andersson *et al.* [3]. In this protocol the native protein is electrophoresed for an extended time (24 h) under non-denaturating conditions on a polyacrylamide gradient gel. The protein migrates until it reaches the pore exclusion limit, which is to a first approximation dependent on the size of the protein. The recombinant HSF migrates on such a gel with an estimated size of 290 kDa consistent with an oligomeric state of a trimer (Fig. 5B).

The oligomeric nature of cloned HSF was confirmed by chemical cross-linking. Purified HSF was cross-linked with limited amounts of the bifunctional reagent EGS [1] and analysed on an SDS gel. The major species of cross-linked HSF migrates with an estimated size of 280 kDa, in addition to larger species at the limiting mobility of the gel (Fig. 5A). The same result was achieved with a 100-fold dilution of cloned HSF protein suggesting that the oligomerization of HSF to trimers is not due to an artificially high concentration of the recombinant protein.

Discussion

There are four characteristic criteria by which heat shock factors are commonly identified, namely the existence of a conserved DNA-binding domain (indicated by amino acid homology), a putative oligomerization domain (indicated by structural motifs of leucine zippers), the ability of the protein to bind heat shock elements and finally the tendency of the protein to form oligomers [24, 27, 28, 37]. Here we demonstrate that these criteria are fulfilled for *Athsf1*, the first HSF gene from *Arabidopsis*. Plant HSF genes have been previously isolated as cDNAs from tomato [29] but *Arabidopsis* is the first organism for which cDNA and corresponding genomic sequences have been described (this paper), and the only plant species whose recombinant HSF seems to exist as a multimeric complex upon expression in *E. coli*.

The comparison of the amino acid homologies between *Athsf1* and other cloned heat shock factors from tomato, *Drosophila* (Fig. 6A), *S. cerevisiae*, *K. lactis*, man, mouse and chicken reveals that the highest similarity is found in the DNA-binding domain: 93% between *Athsf1* and *Lphsf8*, followed by *Lphsf24* (77%) and *Lphsf30* (71%). *Lphsf8* is constitutively expressed in tomato, whereas *Lphsf24* and *Lphsf30* show increased mRNA levels after heat shock [29]. The DNA-binding domain of Athsf1 is interrupted by an intron of 148 nucleotides. The consensus nucleotides at the borders and the high AT content of 69% are in good agreement with published plant introns [9, 32].

Additional sequences that exhibit significant homology with HSFs from other species are only located in the leucine zipper motifs implicated in oligomerization of *Drosophila* and *S. cerevisiae* HSF [34]. A fourth leucine zipper motif is located in the carboxy-terminal region (Fig. 6B). In higher eukaryotes, this C-terminal leucine zipper is required to maintain HSF in a monomeric and non DNA binding state under non-heat shock conditions [24]. In contrast to the situation in higher eukaryotes disruption of the C-terminal zipper in yeast has no observable regulatory effect, although, the leucine zipper motifs are located within the transactivator domains [5]. The activator domain of tomato HSF was investigated by deletion and a substitution analysis [36]. For *Lphsf8*, which shows the highest homology to *Athsf1*, a 50 amino acid C-terminal activator domain containing two Trp elements is described. These alternative operating elements constitute the functional center of the activator domain. The only similarity between *Athsf1* and *Lphsf8* in the C-terminal part of the protein is a short match in

Fig. 6. Conservation of HSF sequences. A. Amino acid homologies between DNA-binding domains of *Athsf1*, tomato *Lphsf8*, *Lphsf24*, *Lphsf30* and *Drosophila Dmhsf*. B. Conserved heptad repeats in leucine sipper motifs of *Athsf1*, *Lphsf8*, *Lphsf30* and *Drosophila Dmhsf*. Heptad repeats of hydrophobic amino acids are marked by dots and asterisks.

one of this Trp elements (*Lphsf8* 467–482, *Athsf1* 432–448); this structure may be also a candidate to modulate the transcription stimulating activity of *Athsf1*. Although only the *Athsf1* cDNA was identified by screening with heterologous tomato gene probes *Athsf1* may not be the sole heat shock factor in *Arabidopsis*. Firstly, the low-stringency Southern blot hybridization gave rise to at least two additional cross-hybridizing fragments. Secondly, clones derived from the genomic library screen contain sequences with homology to C-terminal parts of the DNA-binding domain, in addition to leucine zipper motifs reminiscent of the oligomerization domain. However none of these genomic sequences was represented in the cDNA library and the gene structure could not be determined unambigously (data not shown). If other HSF genes are functional in *Arabidopsis* their expression would be much less abundant than that of *Athsf1*. The northern hybridization shown in Fig. 3 appears to be specific for *Athsf1* mRNA since an oligonucleotide probe derived from the 5′-untranslated leader, a highly divergent region among HSF, was used. The increase of the signal upon rehybridization with the whole cDNA cannot exclude the possibility that mRNAs of similar size from other HSF genes cross-hybridize due to their homology in the DNA-binding domain. A clear discrimination would only be possible with gene-specific cDNA probes from other potential HSF genes from *Arabidopsis*. It seems also possible that other HSFs are expressed under heat shock conditions or in certain developmental stages and for that reason are not represented in our cDNA library. A developmental expression of Hsp is indicated by the detection of small Hsp in *Arabidopsis* seeds [11, and unpublished results]. Small Hsp were also found in other plant species in different developmental stages [10, 17, and unpublished results] but it is not yet known whether HSF is involved in developmental expression.

The expression of HSF in animals seems to be constitutive but its ability to bind DNA is factor-specific and partially regulated by heat (see [33] for review). The level of *Athsf1* mRNA in *Arabidopsis* increases two-fold upon heat shock. A heat-inducible increase in mRNA levels have also been described for two tomato HSF genes but not for *Lphsf8* [29], the gene which shares the highest sequence similarities with *Athsf1*. The lack of HSEs in the promoter region of *Athsf1* suggests a post-transcriptional mechanism controlling the

mRNA stability. However, it cannot be excluded that other unknown elements could stimulate heat-inducible transcription. The putative promoter region contains two CCAAT box sequences, but no other known promoter elements including GC boxes and the classical TATAAA motif were identified. Although approximately 20% of eukaryotic promoters lack typical TATA box elements, the transcriptional initiation of these mRNAs is not yet understood and cannot be generalized at all [2]. GA elements located downstream of the transcription start point are reminiscent of similar elements found in promoters of *Drosophila* heat shock and histone genes. It has been shown that the GAGA factor is constitutively bound to these elements and a potential role for this protein in displacing or altering nucleosomes *in vivo* has been suggested [11, 19].

In unstressed cells of *Drosophila* HSF is present in a monomeric form with no DNA-binding activity and in response to heat HSF assembles into a trimer, that binds the HSE [37]. The active form of HSF *in vivo* is still unknown, but recombinant HSF of *Drosophila* expressed in *E. coli* shows constitutive DNA-binding properties and is present as a multimer indicated by gel retardation assays and cross-linking experiments [7]. *Athsf1* expressed in *E. coli* at 37 °C, the hs temperature of *Arabidopsis*, is present in a trimeric form and binds a synthetic HSE oligonucleotide. The active state of ATHSF1 may be a consequence of either the high growth temperature of *E. coli* and/or the expression in a recombinant system. It is not yet known whether the authentic *Arabidopsis* HSF is trimerized and activated only after heat shock. A more sensitive detection by specific antibodies or a transgenic overexpression of *Athsf1* will be required to verify the elucidated *in vitro* properties of the recombinant protein.

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