Identification of an *Arabidopsis thaliana* cDNA encoding a HSP70-related protein belonging to the HSP110/SSE1 subfamily

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Abstract Heat-shock protein 70 (HSP70)-related proteins are classified in two main subfamilies: the DnaK subfamily and the HSP110/SSE1 subfamily. We have characterized the first plant member of the HSP110/SSE1 subfamily, HSP91. At least two, tightly linked genes encoding HSP91 are present per haploid *Arabidopsis* genome. HSP91 is constitutively expressed in non-stressed *Arabidopsis* plants and is transiently induced by heat shock.

Key words: Heat shock; HSP110/SSE1 subfamily; (*Arabidopsis thaliana*)

1. Introduction

Exposure of all living cells to heat shock or to other environmental stresses results in the synthesis of a set of specific proteins known as heat-shock proteins (HSPs). In several cases, these proteins have been shown to contribute to tolerance to elevated temperatures [1]. Most HSPs are also expressed in unstressed cells, indicating that they play an essential role in the maintenance of normal cell functions as well. The most important function of the HSPs is as molecular chaperones in protein folding and assembly in multimeric complexes (for a review, see [2–5]).

HSPs are subdivided into several classes according to their molecular mass. The 70-kDa family of HSPs (HSP70) is a major, well characterized, group of molecular chaperones. HSP70s are abundant in the cell and execute many functions of pivotal importance including binding of nascent polypeptide chains in ribosomes, maintaining endoplasmic reticulum and mitochondrial protein precursors in translocation-competent conformation, blocking non-productive protein interactions, and modulating the oligomeric state of macromolecular assemblies [5].

The essential functions of HSP70s determine their ubiquity and the stringent evolutionary conservation. For example, the *Escherichia coli* DnaK protein shares approx. 50% amino acid sequence identity with mammalian HSP70s. The most striking homology is found in the N-terminal two-thirds of the molecule, an ATP-binding domain. The C-terminal third is more divergent and is believed to bind unfolded proteins [3]. In plants, several cDNAs and genes coding for HSP70s have been studied and all have characteristic features conserved between bacterial, yeast and animal HSP70s [6–9].

Recently, our knowledge of HSP70s as a highly conserved group of proteins has been challenged by the isolation of two yeast HSP70 genes, *sse1* an *sse2* [10] and the characterization of several animal cDNAs coding for high-molecular weight (HMW) heat shock-inducible proteins (90–110 kDa) with homology to HSP70s [11–13]. Since these proteins have several common features and low homology to the other HSP70s, they were proposed to represent a new HSP110/SSE1 subfamily of HSP70-related proteins [11]. The 'typical' HSP70s were placed in the DnaK subfamily. Lately, a cDNA encoding a 170-kDa glucose-regulated stress protein (GRP170) from Chinese hamster ovary cells has been cloned [14]. It seems to be the most distant member of HSP70-like proteins. GRP170 shares common features with both of the above mentioned groups and is placed in a separate group together with a putative protein encoded by an unidentified open reading frame from *Caenorhabditis elegans* [14].

Here, we report the characterization of the first plant cDNA encoding an HMW HSP70-like protein. Computer analysis clearly indicates that the putative polypeptide belongs to the HSP110/SSE1 subgroup. The cDNA corresponds to at least two tightly linked genes which are expressed in non-stressed plants and are transiently induced by heat shock.

2. Materials and methods

2.1. Plant material

For DNA isolation, *Arabidopsis thaliana* (L.) Heynh ecotypes Columbia and C24 were grown in soil at 22°C under a 16 h light/8 h dark regime. For heat shock induction tests, 3-week-old soil-grown plants ecotype Columbia were placed in growth chambers at 22°C (control) and at 37°C. Whole plant samples from control and heat shock-treated plants were collected at different time points, immediately frozen in liquid nitrogen, and subsequently used for RNA isolation. Samples were taken 30, 60, 105, and 180 min after applying the heat shock.

2.2. Sequencing

Both strands of the cDNA were sequenced by the dideoxy chain termination method using a 7-deazaG Kit (US Biochemical, Cleveland, OH) according to the manufacturer's specifications. Initially, a pair of vector primers was used and then gene-specific primers were synthesized on an oligonucleotide synthesizer (Applied Biosystems, Forster City, CA).

2.3. Rapid amplification of cDNA ends (RACE)

Amplification of the 5'-end of mRNA was carried out according to Troutt et al. [15]. The first strand of the cDNA template was synthesized on 5 μg of total leaf RNA using a preamplification kit (Gibco/BRL, Gaitherburg, MD) according to the manufacturer's instructions.

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Abbreviations: HMW, high-molecular weight; HSP, heat-shock protein; PCR, polymerase chain reaction; 5' RACE, rapid amplification of 5' cDNA ends; SDS, sodium dodecyl sulfate

The nucleotide sequence report here has been deposited in the EMBL Database under accession number Z70314.

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2.4. DNA and RNA blotting hybridizations

Total plant DNA and RNA were isolated according to standard procedures [16,17]. RNA samples were resolved by glyoxal/dimethylsulfoxide electrophoresis with modifications [18]. DNA and RNA samples were blotted on Hybond N membranes (Amersham, Aylesbury, UK) according to the manufacturer's instructions. Equal loading of RNA samples was controlled by staining the membrane with methylene blue. DNA and RNA blot hybridizations were performed in 0.25 M Na-phosphate buffer, pH 7.2, 7% (w/v) SDS and 1% (w/v) bovine serum albumin at 65°C overnight [19]. Membranes were washed in 40 mM Na-phosphate buffer, pH 7.2, 1% (w/v) SDS, 4 times for 15 min at 60°C. ^{32}P-labelled (Redivue [α-^{32}P]dCTP, Rediprime DNA Labelling Kit; Amersham) A4 cDNA was used as a probe. For quantitation of radioactivity in the hybridized bands a PhosphoImager 445 SI (Molecular Dynamics, Sunnyvale, CA) was used.

2.5. Computer analysis

For computer analysis, GCG (Genetic Computer Group Inc., Madison, WI, USA) software was used. Multiple sequence alignments were carried out using the PILEUP Program. Aligned sequences were edited using program SeqVu (The Garvan Institute of Medical Research, Sidney, Australia). Pair-wise amino acid sequence similarities were calculated using the GAP program. Dot-matrix comparison of the sequences was performed by COMPARE and DOTPLOT programs with window 30 and stringency 22.

3. Results

3.1. Characterization of A4 cDNA from Arabidopsis

After screening an *A. thaliana* cDNA library expressed in yeast for the tolerance to oxidative stress [20,21], several cDNAs had been isolated for which, on retesting, the provided tolerance had not been confirmed (Babiychuk et al., unpublished data). One of them (the A4 clone) showed homology to the ATP-binding domain of HSP70 proteins. The cloned cDNA was 2.7 kb in length and hybridized a mRNA of similar length (Fig. 1A). Sequencing revealed that the cDNA had a single 2424-bp contiguous open reading frame corresponding to 808 amino acids followed by a TAA stop codon and a poly(A) tail at the 3′-end. Because of the lack of an ATG codon at the 5′-end of the cDNA, the 5′-end of the corresponding mRNA was amplified using PCR-RACE

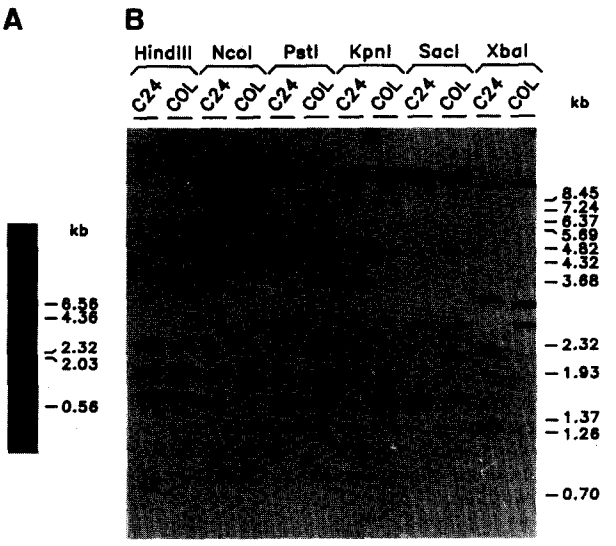

Fig. 1. (A) RNA gel blot hybridization of total mRNA from unstressed plants with labelled A4 cDNA. (B) Southern blotting of total DNA samples from *A. thaliana* ecotypes Columbia and C24 with labelled A4 cDNA.

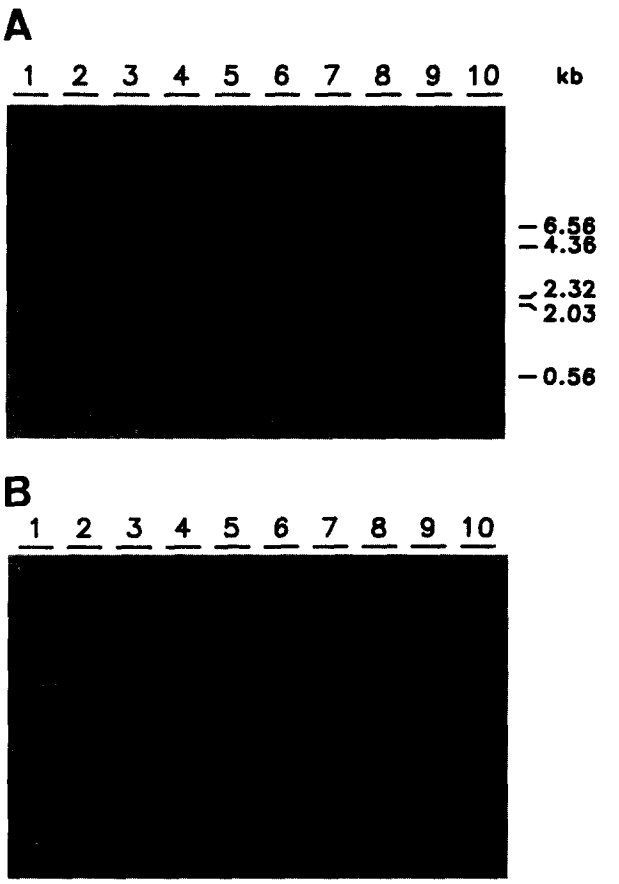

Fig. 2. (A) Heat shock-inducible accumulation of A4 mRNA. 3-week-old, soil-grown plants were transferred to growth chambers at 22°C (lanes 1–5) or at 37°C (lanes 6–10) and RNA samples taken after 0 min (lanes 1,6), 30 min (lanes 2,7), 60 min (lanes 3,8), 105 min (lanes 4,9), and 180 min (lanes 5,10) were used for RNA gel blot analysis with A4 cDNA as probe. (B) The membrane was stained with methylene blue to confirm equal RNA loading.

[15]. The longest PCR products were cloned and sequenced. Two types of sequences were obtained sharing 95% identity in the coding region, with one of them being identical to the cDNA of interest in the overlapping region. This clone was further analyzed and contained an additional 223 bp and an ATG codon 154 bp downstream of the 5′-end and preceded by three in-frame stop codons. The sequence ACA upstream of the ATG codon, with a purine at the −3 position, was in good agreement with a consensus sequence required for effective translation initiation of plant mRNAs [22]. However, another residue essential for translation initiation, G in position +4 (in 74% of the mRNAs examined), was substituted for an A. Thus, the full-length cDNA of 2866 bp has been reconstituted. It encoded a putative protein of 831 amino acids with a calculated molecular mass of 91.6 kDa and an isoelectric point of 4.9.

Since the PCR-RACE revealed two different sequences with 95% identity in the coding region and with only 53% identity in the 5′-untranslated regions, there might be at least two copies of the corresponding gene. This was confirmed by Southern blot hybridization of total DNA from two *Arabidopsis* ecotypes, Columbia and C24, with the labelled cDNA as a probe (Fig. 1B). Complex hybridization patterns with several bands were obtained after digestion with *Hin*dIII, *Pst*I, *Sac*I, and *Xba*I indicating that more than one copy of

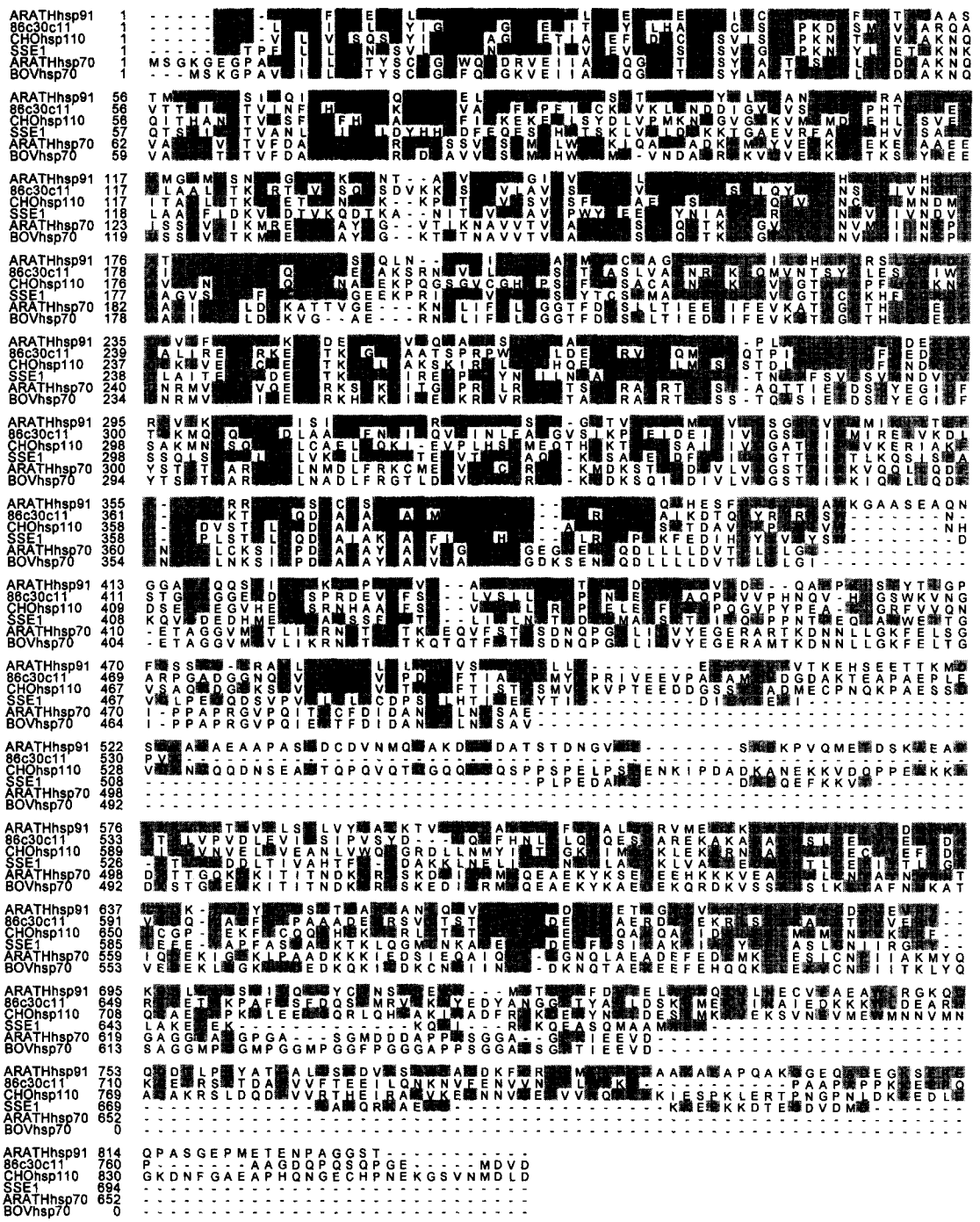

Fig. 3. Comparison of the deduced *A. thaliana* HSP91 amino acid sequence with several members of the HSP110/SSE1 subfamily and with 'typical' HSP70s belonging to the DnaK subfamily. ARATHhsp91, *A. thaliana* HSP91; 86c30c11, unindentified open reading frame 86.4 kDa from *C. elegans* (accession number Q05035); CHOhsp110, HSP110 from Chinese hamster ovary cells (S51311); SSE1, yeast SSE1 protein (P32589); ARATHhsp70, heat shock cognate protein 70-1 from *A. thaliana* (P22953); and BOVhsp70, bovine heat shock cognate protein 70 (X53827). Amino acids identical to HSP91 are shaded. Hyphens depict gaps.

the gene was present in the genome. On the other hand, *Nco*I and *Kpn*I digests gave a single band of a large size. The data were consistent with the presence of two tightly linked gene copies. A RFLP between the two ecotypes was observed.

3.2. Heat shock-inducible expression of A4

Since sequencing revealed homology of A4 to HSP70 proteins, we addressed the question of whether the corresponding gene is induced by heat shock. The results of RNA gel blot analysis of RNA samples from normal and heat shock-stressed *A. thaliana* ecotype Columbia plants are shown in Fig. 2. Constitutive expression of the gene(s) was observed in unstressed plants with strong induction by heat shock at 37°C. The maximum level of the mRNA was observed 60 min after the onset of the heat shock. In 180 min the steady-state mRNA level dropped to the basal level of unstressed plants. Quantitation of radioactivity in the hybridizing bands revealed that the maximal steady-state level of A4 mRNA was

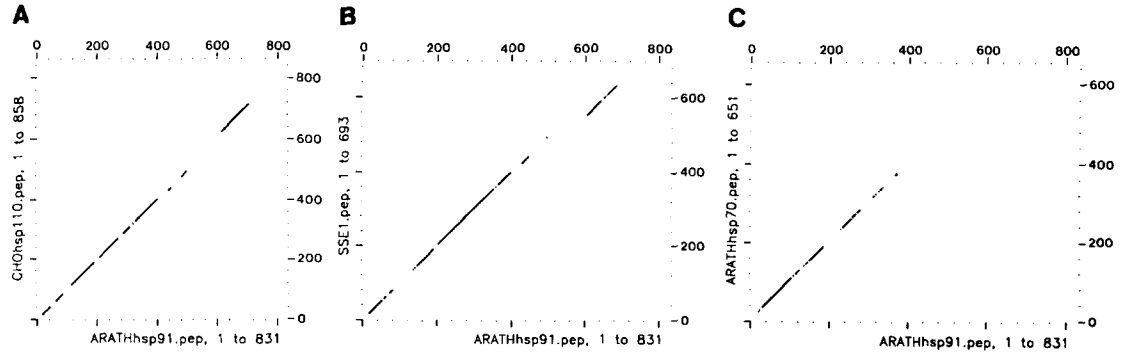

Fig. 4. Dot-matrix comparison of HSP91 versus Chinese ovary cells HSP110 (A), yeast SSE1 (B), and *A. thaliana* HSP70 (C).

at least 10-fold higher during heat shock than under normal conditions. Taking into account the induction by heat shock and the deduced molecular mass of 91.6 kDa of the putative protein, the cloned A4 cDNA was designated as *A. thaliana* HSP91.

3.3. Comparison of the deduced amino acid sequence of A. thaliana *HSP91 to other HSP70-related proteins*

The deduced HSP91 protein sequence was used to search several protein data bases for related sequences using Experimental GENINFO BLAST Network Service (NCBI, Bethesda, MD). The most significant homology was found with a recently identified group of HMW HSP70-related proteins: a hypothetical 86.9-kDa protein encoded by an unidentified open reading frame from *C. elegans* cosmid C30C11 [23], a sea urchin egg receptor for sperm [10], a heat shock protein of 110 kDa from hamster ovary cells [11], mouse heat shock proteins 105a and 105b [13], two unpublished sequences of mouse germ cell-specific protein APG-1 (Kaneko et al., unpublished), and a heat shock protein HSP71E (Morozov and Raychaudhuri, unpublished). The extent of homology of *A. thaliana* HSP91 to all these proteins was between 58 and 62% similarity and 39–42% identity, similar to the homology shared with human HSP70RY [24] and with SSE1 heat shock protein from yeast (Table 1). Recently, the above-mentioned proteins have been proposed to belong to the highly divergent HSP110/SSE1 subfamily of HSP70 proteins. HSP91 appeared to share common characteristics with these proteins (Fig. 3). The N-terminal part (≈first 400 amino acids) of the molecule showed the highest extent of homology (63–67% similarity, 43–51% identity), which is believed to be the ATP-binding domain. It contained five sequence motifs, which formed an ATP-binding pocket and had common features with the ATP-binding domains of HSP70s, actin, and sugar kinases [25]. The N-domain of HSP91 contained all five motifs which fit the deduced consensus sequences on average to 74%. Like other HMW HSP70-related proteins, HSP91 had a higher content of cysteine residues (9) in the N-terminal domain than members of the DnaK subfamily: 2 cysteines for bovine HSP70 and 4 cysteines for the *A. thaliana* HSP70.

The second region of high homology between HSP91 and the HSP110/SSE1 subfamily was found in the C-terminal part of the molecule between residues ≈600 and ≈700 (62–69% similarity, 46–48% identity), which is thought to be a putative protein-binding domain. Another characteristic feature of the HSP110/SSE1 subfamily, not found in members of the DnaK subfamily, was the presence of an 'insertion' sequence with a low extent of homology [11]. HSP91 also contains such an insertion sequence between residues 501 and 575.

In recent studies, the relationship of several plant HSP70s to these subgroups has never been discussed. Therefore, we investigated the relationship of the cloned HSP91 and other plant HSP70s to the established subgroups and to each other. The extent of homology between *A. thaliana* HSP91 and HSP70 was lower (Table 1) than between HSP91 and all of HSP110/SSE1 proteins. At the same time, the homology between *A. thaliana* HSP70 and bovine HSP70 was very high. The results of dot matrix analysis revealed homology of HSP91 to HSP110 and SSE1 along the whole sequence, whereas in the case of the *A. thaliana* HSP70 the homology

Table 1
Sequence similarities and identities of HSP70-like proteins

	ARATH HSP91	C30C11	HUM 70RY	SUSPE	CHO HSP110	MUS HSP105	SSE1	ARATH HSP70	BOV HSP70
ARATH HSP91	–	41	42	40	39	39	40	34	32
C30C11	63	–	47	42	44	44	38	30	31
HUM 70RY	61	65	–	51	64	66	40	33	30
SUSPE	58	64	66	–	38	47	35	31	30
CHO HSP110	60	63	78	63	–	96	40	31	31
MUS HSP105	60	62	79	64	98	–	41	32	32
SSE1	62	59	59	57	59	61	–	28	28
ARATH HSP70	56	53	56	54	56	57	51	–	76
BOV HSP70	56	52	53	52	56	57	51	85	–

Percentages of identities are above the diagonal and percentages of similarities are under the diagonal. For accession numbers, see legend to Fig. 5. ARATH, *A. thaliana*; C30C11, *C. elegans* cosmid; HUM, human; SUSPE, sea urchin egg receptor for sperm; CHO, chinese ovary; MUS, mouse; SSE, *Saccharomyces cerevisiae* SSE1 protein; BOV, bovine.

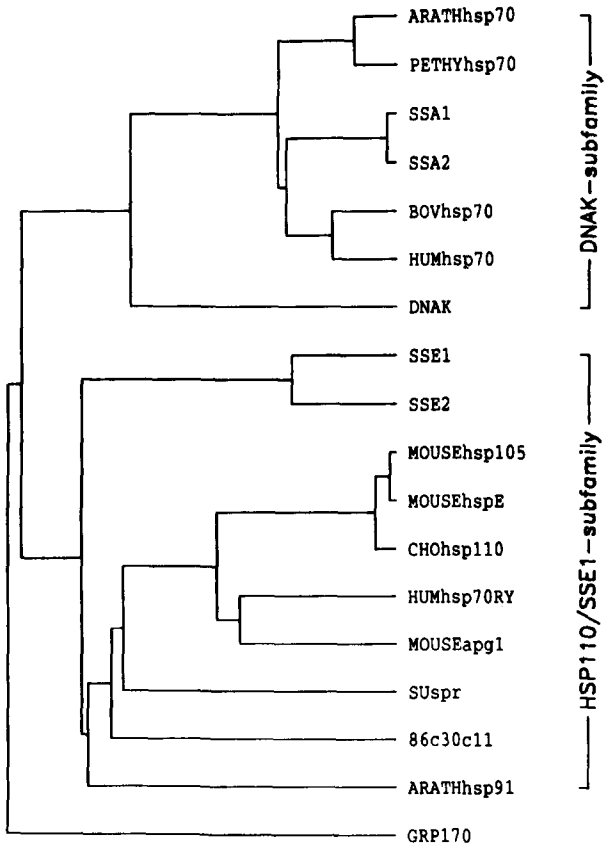

Fig. 5. Dendrogram resulted from multiple alignment analysis of different HSP110/SSE1-related and HSP70 sequences. Abbreviations and accession numbers used are as follows: ARATHhsp70 and PETHYhsp70, heat shock cognate 70 proteins from *A. thaliana* and *P. hybrida* (P22953 and P09189), respectively; SSA1 and SSA2, *S. cerevisiae* heat shock proteins SSA1 and SSA2 (P10592 and P10591), respectively; BOVhsp70, bovine hsc70 (X53827); HUMhsp70, human hsp70 (M11717); DNAK, *E. coli* DnaK (K01298); SSE1 and SSE2, *S. cerevisiae* hsp70-like proteins, (D13908 and D13909), respectively; MOUSEhsp105, mouse heat shock protein 105 kDa (D67016); MOUSE hspE, mouse hsp71E (L40406); CHOhsp110, Chinese hamster ovary cells hsp110 (S51311); HUMhsp70RY, human hsp70RY (P34932); MOUSEapgl, mouse testis-specific protein APG-1 (D49482); Susperm, sea urchin egg receptor for sperm (L04969); 86c30c11, hypothetical 86.9-kDa protein from *C. elegans* cosmid C30C11 (Q05036); GRP170, glucose-regulated 170-kDa heat shock protein from Chinese hamster ovary cells (U34206); ARATHhsp91, *A. thaliana* HSP91 (Z70314).

was observed only in the N-terminal domain (Fig. 4). To study further this relationship, a multiple alignment analysis of a number of HSP-related protein sequences was carried out. A dendrogram of the analysis is presented in Fig. 5. All the sequences analyzed could be divided into two large groups, the HSP110/SSE1 and DnaK groups. HSP91 fell into the HSP110/SSE1 subfamily, whereas earlier cloned plant HSP70s belong to the DnaK group. The recently cloned, glucose-regulated protein GRP170 did not belong to any group and formed a separate branch.

4. Discussion

In this study we have characterized an *A. thaliana* cDNA encoding a putative novel heat shock-inducible protein. Following the established principle of HSP classification by the apparent molecular mass, this protein must belong to the HSP90 class. All known members of the HSP90 class are highly conserved [1] and were found in association with tyrosine kinases and steroid hormone receptors probably serving regulatory functions [2–5]. However, analysis of the deduced amino acid sequence of HSP91 did not reveal any homology to the members of the HSP90 class. We also failed to find any homology of HSP91 to another HMW heat-shock protein, namely HSP104, which has been shown to be practically indispensable in acquiring inducible thermotolerance by yeast cells [26]. Several plant genes coding for HMW HSPs have previously been described, However, all of them fall into the HSP90 [27–30] or HSP104 class [31,32]. Thus, HSP91 is a new plant heat shock-inducible protein.

Surprisingly, HSP91 shows considerable homology to several recently cloned cDNAs encoding HMW HSP70-like proteins of animal origin and to two yeast HSP70-related proteins, SSE1 and SSE2. These proteins have homology to HSP70s, but possess, in addition, specific features placing them in a separate HSP110/SSE1 subfamily. Computer analysis clearly showed that HSP91 can be classified in this group: (i) it has a high molecular mass; (ii) amino acid sequence homology of HSP91 is pronouncedly higher to the members of this group than to the typical members of HSP70; (iii) it contains the insertion sequence lacking in HSP70s; (iv) it has a high homology to the HSP110/SSE1 subfamily in the C-terminal region; (v) it is characterized by a higher content of Cys residues in the N-terminal domain compared to HSP70s. The simplified phylogenetic tree obtained by multiple sequence alignment analysis reflects the relationship between several members of the HSP110/SSE1 and DnaK subfamilies. It is worthwhile noting that the HSP110/SSE1 subfamily is more divergent than the DnaK subfamily.

HSP91 is constitutively expressed in non-stressed *Arabidopsis* plants and is transiently induced by heat shock. Such transient expression has not been found for the typical HSP70s or for the members of the HSP110/SSE1 subfamily studied to date. However, a similar expression pattern was observed for HSP95 in a tomato suspension culture [33]. The maximal level of HSP95 was observed approx. 90 min after administration of heat shock, falling to about 30% of the maximum after 240 min.

We can only speculate about the possible functions of the HSP91. Strong induction by heat shock suggests a role in thermotolerance. Indeed, expression of an animal homologue, HSP110 from hamster ovary cells, is strongly correlated with thermotolerance [34]. On the other hand, a relatively high level of basal expression in unstressed cells could be related to a putatively important function of the HSP91 under normal conditions. The presence of an N-terminal ATP-binding domain and a more variable domain probably involved in peptide binding suggests that HSP91 acts as a chaperone. Matching of the ATP-binding motifs of HSP91 to consensus sequences (74%) is comparable to that of bovine hsp70 (81%) and other HSP70-related proteins. Thus, HSP91 probably requires ATP for exerting its function. The high Cys content in the N-terminal domain of the molecule suggests a possible dependence of the activity on the cellular redox state.

In animals, the members of the HSP110/SSE1 group appear to serve very diverse functions and have different locations in the cell. Chinese hamster HSP110 is localized to the nucleoli in both stressed and unstressed cells and is inferred to participate in ribosome assembly [35]. In contrast, mouse HSP105

has never been found in nucleoli and is localized in the cytoplasm and nuclei [36]. The other product of the *hsp105* gene, a 90-kDa protein, is synthesized only after continuous incubation of murine cells at 42°C. The corresponding mRNA is produced by alternative splicing of the primary transcript [13]. The function of the yeast SSE1 and SSE2 proteins is still unknown. Disruption of the *sse1* gene results in slow-growing cells at any temperature, suggesting an important role under normal conditions [10]. The most distant member of the group, a sea urchin sperm receptor, is located on the egg surface and interacts with the sperm protein, bindin [12]. Since the putative ATP-binding domain of HSP91 starts right from the N-terminus, the protein probably has a cytoplasmic or nuclear location. The sequence KKKVKK found at position 576 of the HSP91 amino acid sequence possibly serves as a nuclear localization sequence [37]. Further analysis of HSP91 will reveal its function in non-stressed plants and its possible role in protecting cells from adverse environmental conditions.

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