Plant Stress ©2008 Global Science Books

Membrane-based Activation of HSFs by Heat Shock in Tobacco Cells

Ahmed Mansour[1*] • Ahmed Shawky[1] • Rajinder Dhindsa[2]

[1] Genetics Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt
[2] Department of Biology, McGill University, Montreal, Canada

Corresponding author: * **alzohairy@yahoo.com** or **amansour@zu.edu.eg**

ABSTRACT

The range of temperature fluctuation which a plant can withstand depends on its genotype and on the available time to acclimatize to novel temperature. The plant responses are entirely mediated by signaling processes by which the plant can sense the changes in the environment and signal its genes to respond. A family of nuclear-encoded *trans*-acting transcription factors, termed the heat shock factors (HSFs), governs the expression of heat shock proteins (HSPs), which in turn, protect plant from heat shock effects. The enhanced expression of HSPs is regulated by heat shock factors (HSFs). Plant HSFs are structurally complex and they are typically composed of multiple exons and introns in the encoding region. In the present investigation, a 7-day old tobacco (*Nicotiana tabacum* cv. 'Bright Yellow 2') cell culture was used to investigate the membrane-based heat activation of HSFs under heat shock. A time course study of HSF1 and HSF2 accumulation using immunoblotting was used to study the synchronization of both HSFs activation in the cells. The effect of membrane fluidity and cytoskeleton reorganization in activating and accumulation of HSF1 and HSF in tobacco cells were demonstrated. In addition, the role of the Heat Activated MAP Kinase (HAMK) in mediating the activation of HSFs was also examined. Comparative analysis of tobacco HSFs and their counterparts in different organisms revealed a high degree of similarities in a corresponding domain, indicating similar function.

Keywords: HAMK, heat shock factor, heat shock, membrane fluidity, tobacco BY-2 cells
Abbreviations: ATP, adenosine triphosphate; **BSA**, bovine serum albumin; **BY-2**, bright yellow 2; **DTT**, dithiothreitol; **EDTA**, ethylene diamine tetraacetic; **EGTA**, ethylene glycol-*bis*(b-aminoethyl ether)*N*',*N*',*N*',*N*'-tetraacetic acid; **ERK**, extracellular signal regulated protein kinase; **HAMK**, heat activated MAP kinase; **HSE**, heat shock elements; **HSF**, heat shock factor; **HSP**, heat shock protein; **MAPK**, mitogen activated protein kinase; **PAGE**, polyacrylamide gel electrophoresis; **SDS**, sodium dodecyl sulfate

INTRODUCTION

Heat shock response is induced in different organisms as diverse as bacteria, fungi, plants and animals by sudden increases in temperature, and is characterized by elevated synthesis of a set of proteins called heat shock proteins (HSPs) (Baler *et al*. 1993; Pirkkala *et al*. 2001; Richter and Buchner 2001). Plants contain highly complex multigene families encoding heat shock factors (HSFs) which bind specifically to heat shock promoter elements (HSE), which are palindromic sequences rich with repetitive purine and pyrimidine motifs (Clos *et al*. 1990; Wu 1995; Nover *et al*. 2001). Promoters of eukaryotic heat shock (hs)-inducible genes share common HSF recognition elements with the palindromic consensus sequence (AGAAn)(nTTCT) located within a few hundred base pairs of the 5′ flanking regions of heat shock genes (Pelham 1982; Pelham and Bienz 1982; Nover 1987; Prandl *et al*. 1997; Schoffl *et al*. 1998). Deletion analyses of plant small HSPs (sHSP) promoters initially pointed out that the *cis*-elements necessary for the heat shock response were also required for developmental regulation (Coca *et al*. 1996; Prändl and Schöffl 1996).

In turn, HSPs act as molecular chaperones and play a central role not only in protection against stress damage but also in folding, intracellular distribution and protein degradation (Baler *et al*. 1993; Richter and Buchner 2001). HSPs comprise several evolutionarily conserved protein families, such as HSP100, HSP90, HSP70, HSP60 and sHSPs (Morimoto 1998).

Sequencing of the *Arabidopsis* genome revealed a unique complexity of the HSF family with 21 members, in contrast to yeast and *Drosophila* with one HSF and vertebrates with four HSFs (Wu 1995; Nover *et al*. 2001). Analyses of expressed sequence tag (EST) libraries revealed that the size of the HSF family is also comparable in other plants, with 17 HSFs thus far identified from tomato ESTs (Nover *et al*. 2001).

In mammals, HSFs play an important role in both heat response and development (Takaki *et al*. 2007). For instance, HSF1 was shown to be the predominant heat inducible HSFs (Murapa *et al*. 2007), while HSF2 responds to developmental signals (Baler *et al*. 1993).

Phosphrylation of HSFs play a major role in its activation. For instance, serine phosphorylation of Hsf1 was shown to be an important determinant of the trans-activating potency, in addition, the latent form of mammalian Hsf1 under normal conditions was shown to be constitutively phosphorylated (Pirkkala *et al*. 2001). Recently, it was shown that activation of Protein Kinase A (PKA) mediates the suppression of an intermediary protein kinase, extracellular Regulated protein Kinase (ERK)1/2, which phosphorylates and suppresses the activation of the heat shock transcription factor 1 (HSF1) (Melling *et al*. 2007).

In that regard, mitogen activated protein kinases (MAPKs) are believed to be ubiquitously involved in signal transduction during eukaryotic responses to diverse stimuli. The involvement of MAPK cascades in signaling various, biotic and abiotic, stresses is well established (Zhang and Klessig 2001; Zwerger and Hirt 2001). For instance, a cold shock-activated MAP kinase (SAMK) has been identified and well characterized (Jonak *et al*. 1996). A heat shock-activated MAP kinase (HAMK) has been identified in alfalfa

Received: 28 July, 2008. Accepted: 16 October, 2008.

Original Research Paper

(Sangwan *et al*. 2002; Sangwan and Dhindsa 2002; Suri and Dhidsa 2008) and tomato (Link *et al*. 2002) cells. Although HAMK has yet to be purified and characterized at the molecular level, it is immunologically related to ERK superfamily of protein kinases. Thereby, anti-phosphoERK (anti-pERK) antibody recognizes the activated form of HAMK (Sangwan *et al*. 2002; Sangwan and Dhindsa 2002). Although the sequence of upstream events leading to the heat-activation of HAMK has been examined, their downstream targets and possible roles in heat-specific gene expression are presently unknown.

In the present investigation, we studied the time course of HSF1 and HSF2 which control the expression of HSPs and examined their activation by HAMK. We showed that membrane fluidity and cytoskeleton remodeling play an important role in heat signaling and HSF activation. Exploring the structural similarity between NtHSF1 and NtHSF2 with their counterparts in different organisms showed similar domains in different species indicating similar function.

MATERIAL AND METHODS

Plant cell suspension

One-week-old tobacco BY-2 (*Nicotiana tabacum* cv. 'Bright Yellow 2') suspension culture cells were grown in 250 ml Erlenmeyer flasks containing 50 ml of MS basal salts media (Murashige and Skoog 1962) (Sigma Chemicals Co., Oakville, Canada) supplemented with 2.72 mM KH2PO4, 100 mg.l^{-1} *myo*-inositol, 1 mg.l^{-1} thiamine, 3 % (w/v) sucrose and 0.9 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The pH of the media was adjusted to 5.0 prior to autoclaving. Cultures were incubated on a rotary shaker at 130 rpm with a 16-h photoperiod at a light intensity of 40 μmol m^{-1} sec^{-1} at 25 ± 0.5°C and 60% relative humidity. Cells were subcultured every 7 days.

Cell viability

Cells from the control and variously treated samples were tested for their viability by using tetrazolium chloride (TTC) (Towill and Mazur 1974) and optical density was measured at 400 nm. For comparisons among several treatments, three to five replicates of each were stained and then the absorbance data was statistically analyzed. All data obtained in the experiment was subjected to analysis of variance according to Snedecor and Cochran (1967) and the comparison of means was done using the LSD test at the 0.05 level of probability, as mentioned by Cochran and Cox (1957).

Heat treatment

Control and variously pre-treated cells were harvested and subjected to heat shock by adding them to 5 mL of MS medium pre-heated and maintained at 37°C in a water bath. After the heat shock treatment, the cells were immediately harvested by vacuum filtration, frozen in liquid nitrogen and stored at -80°C until use. All experiments were repeated at least three times and yielded similar results.

Protein extraction and quantification

The frozen tissue (250 mg) was grounded in liquid nitrogen and total protein was extracted in 400 μl of the extraction buffer (100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 5 mM EDTA, 10 mM DTT, 50 mM β-glycerophosphate, 5 μg ml^{-1} antipain, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin, 10% (v/v) glycerol and 7.5% polyvinylpolypyrrolidone). The homogenate was centrifuged at 13,200 rpm (~20,000 × *g*) for 20 min at 4°C. Supernatant was quickly frozen in liquid N_2 (l) and stored at –80°C until analysis. The protein was quantified by using the Bradford dye-binding assay (BioRad laboratories, Hercules, USA) with BSA as a standard.

Immunoblot analysis for HSFs

Proteins in cell lysates were resolved in a 10% SDS-PAGE gel and electro-blotted onto Hybond™-C Extra nitrocellulose membranes (Amersham Bioscience, Baie d'Urfe, QC, Canada). Membranes were blocked at RT with 3% (w/v) BSA in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA pH 8.0 and 0.1% (w/v) Tween-20) followed by incubation with the primary antibody for 60 min at a 1: 1000 dilution. Primary antibodies used were anti-HSF1 antibody (Cell Signaling Technology, USA) to visualize the accumulation of HSF1, and anti-HSF2 antibody to monitor the level of HSF2 protein accumulation. Membranes were washed in TBST and incubated for 50 min with horseradish peroxidase-linked Protein An antibody (Amersham Biosciences Corp, Baie d'Urfe, QC, Canada) diluted in TBST (1: 10,000 dilution). Antigen-antibodies complexes were visualized using the Supersignal® chemiluminescence kit (Pierce Biotechnology Inc, Rockford, USA).

S^{35} labeling of proteins

Tobacco cells were harvested by sieving through Miracloth (Calbiochem-Novabiochem, San Diego, CA, USA). 200 mg of cells were suspended in 1 ml MS medium and 200 uCi S^{35} methionine/ml was added to it and incubated at 37°C for 3 h. For RT control, cells were incubated at RT for 3 h. Media was removed by spinning shortly four times to wash off unincorporated S^{35}-labeled methionine, quickly immersed in N_2 (l) and stored at -80°C until use. Protein was extracted from these cells (as mentioned in the previous section), and separated on 12% SDS PAGE. Proteins were fixed on the gels by treating the gel with a fixative (H_2O + CH_3COOH, 93+7) for 30 min and rinsed with Amplify (Amersham) for 30 min. Gel was vacuum dried and autoradiographed.

Chemical treatments

Cells were harvested aseptically by sieving through Miracloth™ (Calbiochem-Novabiochem) and 250 mg cells were added to each treatment solution (1 ml). The roles of MEK-related MAPK kinase in the heat activation of HAMKs, and accumulation of HSF were investigated. In these experiments, cells were pretreated with the appropriate chemical, mentioned below, for 2 h at 25°C prior to their exposure to heat-shock at 37°C.

Membrane fluidity, cytoskeleton stability

Membrane fluidity and cytoskeleton stability were experimentally modulated as described before (Sangwan *et al*. 2002). Briefly, benzyl alcohol (BA; 20 mM; Sigma, Oakville, ON, Canada) or dimethylsulfoxide (DMSO, 450 mM; Fisher Scientific, Nepean, ON, Canada) were used to fluidize or rigidify the cellular membranes, respectively. Further, jaspakonolide (JK; 25 μM; Molecular Probes, Eugene, OR, USA) and taxol (50 μM; Sigma) were used as stabilizers of microfilaments and microtubules, respectively. Latrunculin B (Lat B; 25 μM; Sigma) and oryzalin (Ory, 100 μM; Sigma) were used to de-stabilize microfilaments and microtubules, respectively.

Bioinformatics analysis

The bioinformatics analysis domains similarities were conducted using analytic software available at: http://www.ncbi.nlm.nih.gov/, http://us.expasy.org/tools/, http://www.bioinformatics.vg/index2.shtml, and http://www.ebi.ac.uk/interpro.

RESULTS AND DISCUSSION

7-days-old culture BY-2 cells are the optimum age

The viability and growth rate of tobacco BY-2 cells under our growth conditions was examined according to Towill and Mazur (1974). Examining the density of cultured cell using a spectrophotometer shown that tobacco BY-2 cells' best growth rate was reached after 7 days' incubation when the color of the culture was bright (yellowish-green; **(Fig. 1A)**. The cell viability test confirmed these results and showed that the cultures were mostly viable after 7 days' incubation (**Fig. 1B**). Thus we concluded that a 7-days-old culture was the optimum age and growth rate for conducting our experiments.

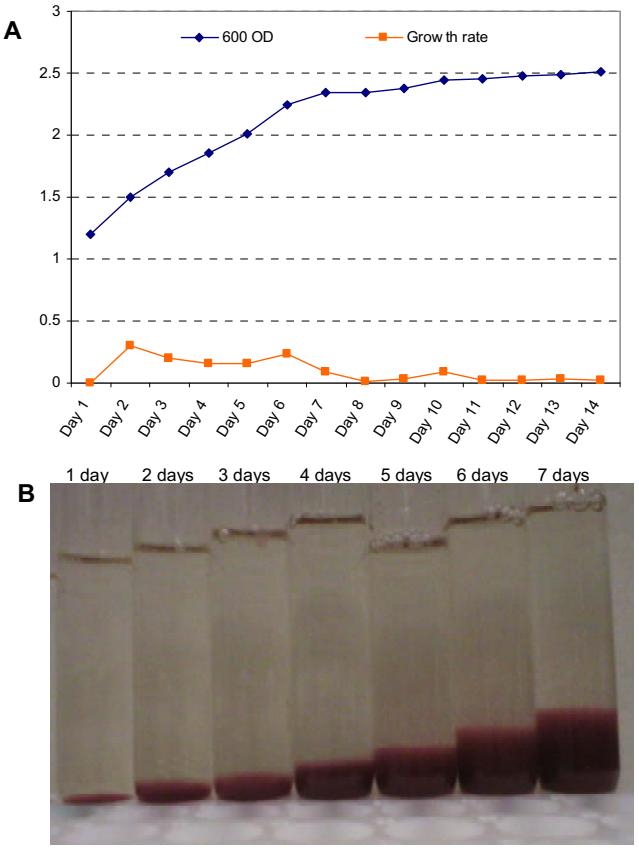

Fig. 1 The growth of tobacco cell cultures on MS media from 1 to 14 days. (**A**) The optimum growth was observed after 7 days using the spectrophotometer, therefore most of our experiments has been done in this age. (**B**) Using cell viability test (TTC) as an indicator for cell growth during 14 days confirmed that 7days old flask was the most viable age for conducting the experiments.

De novo protein synthesis in response to heat shock

After 3 h of exposure to heat stress in tobacco BY-2 cells, HSP70, HSP90, HSP101 and sHSPs were 60% dominant.

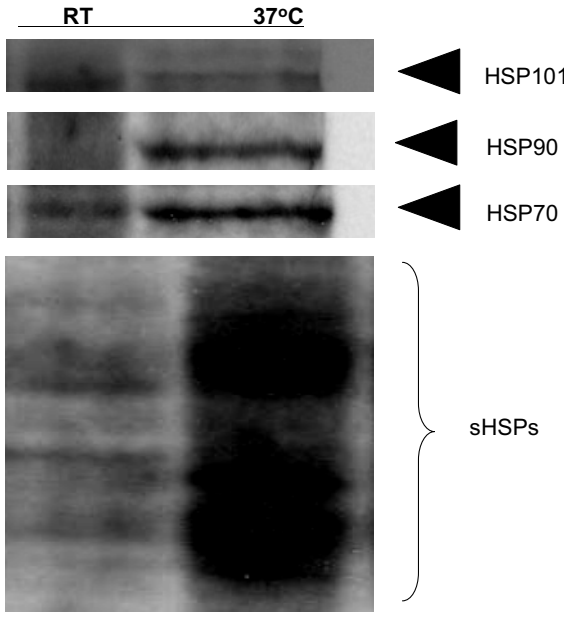

Fig. 2 S^{35} labeling of *de novo* synthesis proteins in response to heat treatment. After heat shock for 3 hours some newly synthesized proteins HSPs is accumulating in the cell to increase the cell tolerance, other proteins disappeared from the gel pattern after the heat shock. HSPs 70, 90, 101 and small HSP27 appear very clearly in the gel pattern.

In agreement to this work hsp90, hsp70, high molecular weight immunophilins and a Hop-like protein have been identified in plant complexes of hsp90 (Owens-Grillo *et al*. 1996; Stancato *et al*. 1996; Reddy *et al*. 1998; Pratt *et al*. 2001; Zhang *et al*. 2003).

In addition, many regular proteins which would normally be found at RT were absent (**Fig. 2**). The dominance of Small HSPs in response to heat shock is in agreement with identified heat dominant proteins indentified in *A. thaliana* genome (Waters *et al*. 1996; Scharf *et al*. 2001). The existence of some small HSPs in both gel patterns, the stressed and non-stressed, indicates their important roles in cell regulation and its important role under normal conditions in folding, intracellular distribution and protein degradation as suggested by Baler *et al*. (1993) and Richter and Buchner (2001).

Time course study of HSFs

Time course accumulations of HSF1 and HSF2 in BY-2 cells were examined. HSF1 accumulation was apparent after 15 min of heat shock and reached a maximum after 2 h (**Fig. 3** upper panel). After 4 h of heat shock no more accumulation of HSF1 was observed. In contrast, some accumulation of HSF2 was observed at RT. Despite the fact that the maximum accumulation of HSF2 was also observed at 2 hours of heat shock, after 4 hours the accumulation of HSF2 was constant (**Fig. 3**, lower panel).

This time course study of NtHSF1 and NtHSF2 showed that NtHSF1 is predominantly heat inducible, while NtHSF2 is expressed in the cell constitutively (**Fig. 3**). These results suggest that NtHSF1 is the predominant heat-inducible HSF, while NtHSF2 responds to the developmental signals, so that it is constitutively expressed in the cells. This hypothetically agrees with what was found in mammalian erythroleukemis cells by Baler *et al*. (1993) findings suggesting the involvement of HSFs in both regulatory processes.

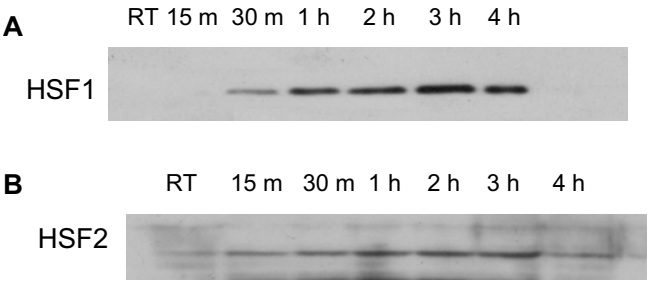

Fig. 3 Time course study for HSF1 and HSF 2 in tobacco cells. (**A**) The cells were exposed to 37°C and samples were collected at the specific time as shown in the Upper panel. (**B**) The accumulation of HSF1 was increased by the treatment and reached its maximum at 2 h. Although HSF2 constitutively present at low levels, there was a considerable increase after 15 min of heat shock reaching the maximum at the same time of 2 h as shown in the lower panel.

Membrane fluidity and cytoskeleton remodeling activate of HSF1

A well known mechanism for temperature signaling is based on temperature-triggered rapid and reversible changes in membrane fluidity (Carratu *et al*. 1996; Örvar *et al*. 2000; Sangwan *et al*. 2002). Relatively little is known about the role of phosphorylation and membrane-based activation of HSFs in the plant heat shock response. It has been previously reasoned that temperature, being a pervasive thermodynamic factor, is sensed at many places in the cell (Kawczynski and Dhindsa 1996). Therefore it was expected that as temperature change spreads over the protoplast and it was also expected that membranes may sense it before it has any effect on cytosolic proteins. Recently, it was reported that Heat-induced HSP70 accumulation requires mem-

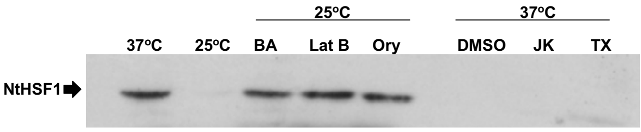

Fig. 4 Modulation of HSF accumulation by chemicals that alter membrane fluidity or cytoskeleton stability. Cells were treated with the membrane rigidifier (DMSO), microfilaments stabilizers (jasplakinolode, JK) and microtubules stabilizers stablizers (taxol) for 2 hours at 25°C and challenged to heat-shock at 37°C for 3 h. The expression of HSF1 protein has not affected. Treatment of cells with plasma membrane fluidizer (benzyl alcohol, BA), microfilaments de-stabilizer (latrunculin B, Lat B) and microtubules destablizer (oryzalin) resulted in accumulation of HSF protein at 25°C.

brane fluidization and cytoskeleton reorganization (Suri and Dhindsa 2008; Mansour 2008). Accumulation of HSFs at 37°C was prevented by chemicals that either rigidify the membrane (DMSO) or stabilize the cytoskeleton (jasplakinolide and taxol) (**Fig. 4**); and HSF1 accumulation was induced at 25°C by chemicals that either fluidize the membrane (BA) or destabilize the cytoskeleton (latrunculin B and oryzalin) (**Fig. 4**).

Our results suggest that chemicals that alter rigidity of the membrane or stabilization of the cytoskeleton affect NtHSF1 accumulation (**Fig. 4**). Thus, as expected, temperature-induced changes in membrane fluidity trigger a cascade of signaling events (Sangwan *et al.* 2002). Therefore, we suggested a causal link between heat membrane fluidity and cytoskeleton reorganization and accumulation of NtHSF1, as a prominent component of the heat shock response in diverse phyla of organisms (Rabindran *et al.* 1991; Baler *et al.* 1993; Murapa *et al.* 2007; Lee *et al.* 2008). Based on these results, it was concluded that the heat-induced accumulation of HSF1, which is the predominant heat-inducible HSF, requires membrane fluidization and reorganization of the cytoskeleton.

MEK inhibitors inhibit the accumulation of pHSF1 and pHSF2

MAP kinases are believed to be ubiquitously involved in signal transduction during eukaryotic responses to diverse stimuli and involvement of MAPK cascades in signaling various biotic and abiotic stresses is now well established (Zhang and Klessig 2001; Zwerger and Hirt 2001). The activation of specific MAPKs is one of many events that occur during the perception and transduction of temperature signals (Sangwan *et al.* 2002). A HAMK has been identified in alfalfa (Sangwan *et al.* 2002; Sangwan and Dhindsa 2002) and tomato (Link *et al.* 2002) cells. The flavone compound known as PD98059 (2'-amino-3'-methoxyflavone) is a potent, selective, cell permeable inhibitor of MEK that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates (Alessi *et al.* 1995; Kultz *et al.* 1998). Likewise, U0126 (1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthiol) butadiene) is a potent and specific inhibitor of MEK1 and MEK2 and the inhibition is non-competitive with respect to MEK substrates, ATP and ERK (de Silva *et al.* 1998; Favata *et al.* 1998). In previous investigation, different concentrations (0-125 μM) of these MEK inhibitors were tested for the inhibition of HAMK activation or HSP70 accumulation (Mansour 2008). In this study, the accumulation of pHSF1 and pHSF2 (phosphorylated form of HSF1 and HSF2) was prevented by using both inhibitors of MEK (a MAPKK), suggesting that the HAMK cascade is an essential upstream component of the heat shock response leading to HSFs' phosphorylation and accumulation in tobacco cells (**Fig. 5**). This suggestion is in agreement with the observation that HAMK can specifically phosphorylate and activates HSF3 *in-vitro* in tomato cells (Link *et al.* 2002).

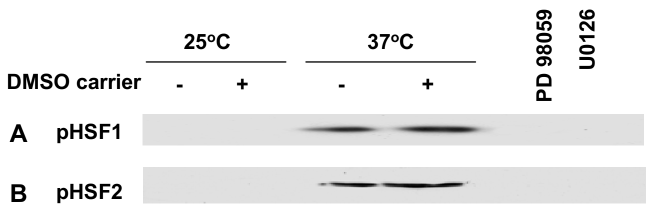

Fig. 5 The treatment of cells with MEK inhibitors strongly inhibited the phosphorylation of (A) pHSF1 and (B) pHSF2.

292 Sequences

NtHSF1

```
MSQRTVPAPFLTKTYQLVDDATTDDVVSWNESGTTFVVWKTAEFAKDLVPTYFKHNNFSSFVRQLNTYGFRKIVPDKWEF   80
ANENFKRGQKELLTAIRRRKTVTPTPAGGKSVVPGTSASPDNSGEDLGSSSTSSPDSKNPGSVDTPGKSQFADLSDENEK  160
LKKDNQMLSSELAQAKKQCDELVAFLNQYVKVAPDMINRIISQGTSGSSYGELVKEVIGGVNDLEAQGSDDDEKGDTLKL  240
FGVLLKENKKKRGPDENADISGSRGKMMKTMDYNLPWMKMSSAPGESNKVCN                              320
.....................TT....S....................................................   80
....................T.T...............S...S......SS.SS..S.......T...S.....S.....  160
............................Y............S...S..SY..................S.......T...  240
....................S...............................                              320
```

Phosphorylation sites predicted: Ser:15 (S), Thr: 6(T), Tyr: 2(Y)

408 Sequences

NtHSF2

```
MDEATCSTNALPPFLTKTYEMVDDPSSDAIVSWSSSNKSFVVWNPPDFARDLLPRYFKHNNFSSFIRQLNTYGFRKVDPE   80
KWEFANEDNFFRGQPHLLKNIHRRKPVHSHSAQNLHGLSSPLTESERQGYKEDIQKLKHENESLHLDLQRHQQDRQGLEL  160
QMQVFTERVQHVEHRQKTMLSALARMLDKPVTDLSRMPQLQVNDRKRRLPGNSCLYNETDLEDTRAISSRALTWENMNPS  240
SLLTINAELLNQLDSSLTFWENVLQDVDQAWIQQNCSLELDESTSCADSPAISYTQLNVDVGPKASDIDMNSEPNANTNP  320
EVAAPEDQAAVAGTTTNVPTGVNDIFWEQFLTENPGSVDASEVQSERKDIGNKKNESKPVDSGKFWWNMKSVNSLAEQLG  400
HLTPAEKT                                                                          480
.........................S.........S............................................   80
............................S.............T.S...................................  160
.................T.................................................S............  240
....................................S.....S.S...S................S..............  320
..............TT....................S...S...S................S...........S......  400
..T.....                                                                          408
```

Phosphorylation sites predicted: Ser: 15 (S), Thr: 5 (T), Tyr: 0 (Y)

Fig. 6 The predicted phosphorylation sites in tobacco NtHSF1 and NtHSF2 sequence using computer based software NetPhos 2.0. Phosphorylation sites predicted, Serine (S), Threonine (T) and Tyrosine (Y).

In silico prediction of possible phosphorylation sites of NtHSF1 and NtHSF2

Phosphrylation of HSFs play a major role in its activation. For instance, serine phosphorylation of Hsf1 was shown to be an important determinant of the trans-activating potency, in addition, the latent form of mammalian Hsf1 under normal conditions was shown to be constitutively phosphorylated (Pirkkala *et al*. 2001). This encouraged us to check possible phophorylation sites of NtHSF1 and NtHSF2 as predicted by their structure using computer based phosphorylation prediction software, Netphosphos 2.0 (Blom et al. 1999) (**Fig. 6**).

In this study, possible phophorylation sites of NtHSF1 and NtHSF2 were predicted by their structure using computer-based phosphorylation prediction software, Netphosphos 2.0 (Blom *et al*. 1999) (**Fig. 6**). The *in silico* prediction revealed multiple potential phosphorylated serine(S) and tyrosin (T) sites which could be linked to its activation.

In this regard, it was reported that a subset of phosphorylated sites involved in Hsf1 repression have been identified. To date, a single site only, Ser230, has been linked with inducible transcriptional activity (Holmberg *et al*. 2001). In this regard, the yeast Hsf is also an inducible serine by phosphorylation (Cotto *et al*. 1966). Moreover, it was reported in *Drosophila* that HSFs undergo phosphorylation at some sites and dephosphorylation at others in response to heat shock, however, with no net increase in the steady state level of HSF phosphorylation (Fritsch and Wu 1999).

Similarity analysis of *NtHSFs* conserved domain among different species

The evolutionary process has produced DNA sequences that encode proteins with very specific functions. The power and ease of using sequence information has however, made it the method of choice in modern analysis. Luckily, in agreement with evolutionary principles, scientific research to date has shown that all genes share common elements. Common genetic elements include promoters, enhancers, polyadenylation signal sequences and protein binding sites.

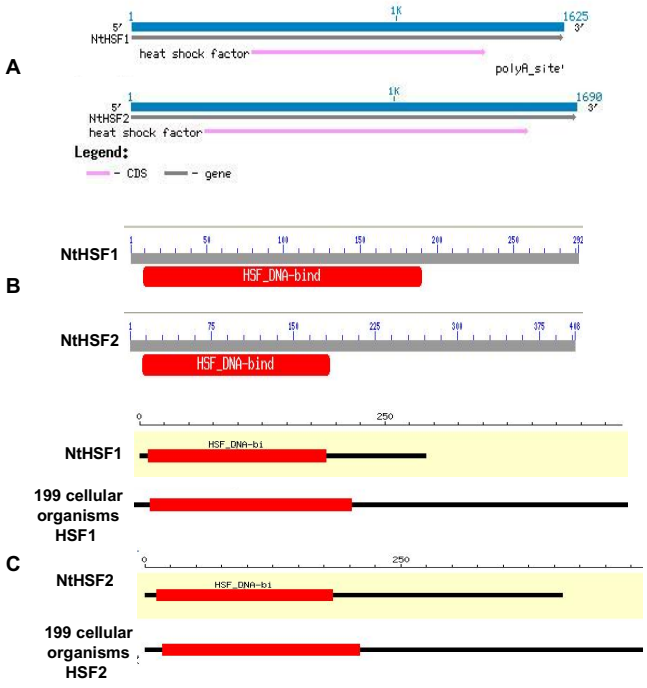

Fig. 7 Blasting of HSF sequences. (**A**) Comparing *Nt*HSF1 and *Nt*HSF2 gene total length. (**B**) comparison of *Nt*HSF1 and *Nt*HSF2 DNA binding domains total length. (**C**) Blasting of tobacco HSF conserved domain with their counterparts in different cellular organisms shows 199 similar domain in eukaryotes.

By comparing the size of both NtHSF1 (292 aa) and NtHSF2 (408 aa) sequences, it was clear that NtHSF2 has more sequences (**Fig 7A**). However, the "DNA binding domain" of NtHSF1 is longer than its counterpart in NtHSF2 (**Fig. 7B**). This domain is functionally important for HSF to bind to DNA and activates Heat shock genes; in addition, it is a common domain in most NtHSFs. By blasting the DNA binding domain of NtHSFs1 and 2 elements with different the available cellular organisms' genomes in NCBI (National Center of Biotechnology Information) genomic resources to date, a similar conserved domain was found among almost 199 of them (**Fig. 7C**). These results suggest that similar function of HSFs could be found in other organisms. The presence of common HSF recognition elements explains the possibility for the same HSFs to be activated between different plants. For instance, it was reported recently that the expression of rice heat shock transcription factor OsHsfA2e enhances the tolerance of transgenic Arabidopsis to environmental stresses in as shown in (Yokotani *et al*. 2007).

Taxonomy display

In this investigation, we aimed to provide a view of the taxonomic range of the sequences associated with NtHSF and the number of sequences associated with each lineage. The expected results of this trial were expected to reveal evolutionary revaltionship and development of HSFs in different taxa. Thus, InterPro© software was used to display the taxonomy of NtHSF (Nicola *et al*. 2007). As shown in **Fig. 8**, the circular display has a taxonomy-tree with the root as its centre, which is NtHSF in this case. Selected model organisms populate the outermost circles. Moreover, nodes of the taxonomy-tree are placed on the inner circles. No significance is attached to the position of the node on a particular inner-circle. However, the nodes themselves are either true taxonomy nodes or artificial nodes created for this display. Furthermore, some protein records may have more than one taxonomy. For example, where a mouse and human sequence have been merged, this will result in multiple taxonomy counts for a protein. The DNA-binding component of HSF lies to the N-terminus of the first nuclear localization signal NLS region, and is referred to as the HSF domain. These results suggest that a similar function of HSFs could be found in other organisms. That explains the possible swapping HSFs between different plants (Yokotani *et al*. 2007).

Cellular localization of NtHSF1 and NtHSF2

Heat shock activation results in relocalisation to the nucleus (Rabindran *et al*. 1991). Using WolFPSORT©, a computer program for the prediction of protein localization sites in cells, it was shown that NtHSF1 exists not only in the nuclease but also in the cytoplasm and chloroplast. However,

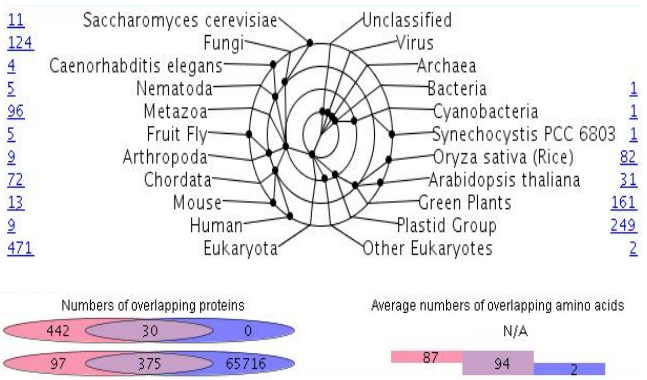

Fig. 8 Taxonomy display of *Nt*HSF providing a view of the taxonomic range of the sequences associated with each *Nt*HSF and the number of sequences associated with each lineage.

NtHSF2 is excited only in the nuclease. This indicates more possible roles of NtHSF1 in the cell (Lee *et al*. 2008). Under normal conditions, HSF is a homo-trimeric cytoplasmic protein, but heat shock activation results in relocalisation to the nucleus (Rabindran *et al*. 1991). Using WolFPSORT©, it was shown that NtHSF1 exists not only in the nuclease but also in the cytoplasm and chloroplast. However, interaction between the N- and C-terminal zippers may result in a structure that masks the NLS sequences: following activation of HSF, these may then be unmasked, resulting in relocalisation of the protein to the nucleus (Schuetz *et al*. 1991).

ACKNOWLEDGEMENTS

The authors would like to acknowledge Bibliotheca Alexandrina – Center for Special Studies and Programs, Grant No. 050176 and the Ministry of higher education, Egypt for their great support during the course of this work.

REFERENCES

Alessl DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *The Journal of Biological Chemistry* **270**, 27489-27494

Baler R, Dahl G, Voellmy R (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Molecular Cell Biology* **13**, 2486-2496

Blom N, Gammeltoft S, Brunak S (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology* **294 (5)**, 1351-1362

Carratu L, Franceschelli S, Pardini CL, Kobayashi GS, Horvarth I, Vigh L, Maresca B (1996) Membrane lipid perturvation modifies the set point of the temperature of heat shock response in yeast. *Proceedings of the National of Academy of Sciences USA* **93**, 3870-3875

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

Coca M A, Almoguera C, Thomas TL, Jordano J (1996) Differential regulation of small heat-shock genes in plants: analysis of a water stress inducible and developmentally activated sunflower promoter. *Plant Molecular Biology* **31**, 863-876

Cotto JJ, Kline M, Morimoto RI (1996) Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. *The Journal of Biological Chemistry* **271**, 3355-3358

DeSilva DR, Jones EA, Favata MF, Jaffee BD, Magolda RL, Trzaskos JM, Scherle PA (1998) Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent energy. *Journal of Immunology* **160**, 4175-4181

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibtor of mitogen-activated protein kinase kinase. *The Journal Biological Chemistry* **273**, 18623-18632

Fritsch M, Wu C (1999) Phosphorylation of *Drosophila* heat shock transcription factor. *Cell Stress Chaperones* **4**, 102-117

Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *The EMBO Journal* **20**, 3800-3810

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) *Proceedings of the National of Academy of Science USA* **93**, 11274-11279

Kawczynski W, Dhindsa RS (1996) Alfalfa nuclei contain cold-responsive phosphoproteins and accumulate heat-stable proteins during cold treatment of seedlings. *Plant Cell Physiology* **37**, 1204-1210

Kultz D, Madhany S, Burg MB (1998) Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. *The Journal of Biological Chemistry* **273**, 13645-13651

Lee YJ, Lee HJ, Lee JS, Jeoung D, Kang CM, Bae S, Lee SJ, Kwon SH, Kang D, Lee YS (2008) A novel function for HSF1-induced mitotic exit failure and genomic instability through direct interaction between HSF1 and Cdc20. *Oncogene* **27**, 2999-3009

Link V, Sinha AK, Vashista P, Hofmann MG, Proels RK, Ehness R, Roitsch T (2002) A heat-activated MAPkinase in tomato: a possible regulator of heat shock response. *Federal European Biochemistry Society Letters* **531**, 179-183

Mansour A (2008) Heat-induced HSP70 accumulation requires membrane fluidization and cytoskeleton reorganization. In: Mansour A (Ed) *Heat-Shock Signaling Transduction in Plant Cells*, VDM, Verlag, Dr Müller, Germany, pp 62-65

Melling CW, Thorp DB, Milne KJ, Krause MP, Noble EG (2007) Exercise-mediated regulation of Hsp70 expression following aerobic exercise training. *American Journal of Physiology Heart Circulatory Physiology* **293**, 3692-3698

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

Murapa P, Gandhapudi S, Skaggs HS, Sarge KD, Woodward JG (2007) Physiological fever temperature induces a protective stress response in t lymphocytes mediated by Heat Shock Factor-1 (HSF1). *Journal of Immunology* **179**, 8305-8312

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-494

Mulder NJ, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Buillard V, Cerutti L, Copley R, Courcelle E, Das U, Daugherty L, Dibley M, Finn R, Fleischmann W, Gough J, Haft D, Hulo N, Hunter S, Kahn D, Kanapin A, Kejariwal A, Labarga A, Langendijk-Genevaux PS, Lonsdale D, Lopez R, Letunic I, Madera M, Maslen J, McAnulla C, McDowall J, Mistry J, Mitchell A, Nikolskaya AN, Orchard S, Orengo C, Petryszak R, Selengut JD, Sigrist CJ, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2007) New developments in the InterPro database. *Nucleic Acids Research* **35 (Database Issue)**, D224-D228

Nover L (1987) Expression of heat shock genes in homologous and heterologous systems. *Enzyme Microbiology Technology* **9**, 130-144

Nover L, Bharti K, Dring P, Mishra SK, Ganguli A, Scharf KD (2001) Arabidopsis and the heat shock transcription factor world: How many heat shock transcription factors do we need? *Cell Stress Chaperones* **6**, 177-189

Örvar BL, Sangwan V, Omann F, Dhindsa RS (2000) Early steps in cold sensing by plant cells: Role of actin cytoskeleton and membrane fluidity. *The Plant Journal* **23**, 785-794

Owens-Grillo JK, Stancato LF, Hoffmann K, Pratt WB, Krishna P (1996) Binding of immunophilins to the 90 kDa heat shock protein (hsp90) via a tetratricopeptide repeat domain is a conserved protein interaction in plants. *Biochemistry* **35**, 15249-15255

Pelham HRB (1982) A regulatory upstream promoter element in the *Drosophila* HSP70 heat-shock gene. *Cell* **30**, 517-528

Pelham HRB, Bienz M (1982) A synthetic heat-shock promoter element confers heat-inducibility on the Herpes simplex virus thymidine kinase gene. *The EMBO Journal* **1**, 1473-1477

Pirkkala L, Nykanen P, Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB Journal* **15**, 1118-1131

Prändl R, Schöffl F (1996) Heat shock elements are involved in heat shock promoter activation during tobacco seed maturation. *Plant Molecular Biology* **31**, 157-162

Pratt WB, Krishna P, Olsen LJ (2001) Hsp90-binding immunophilins in plants: the protein movers. *Trends in Plant Science* **6**, 54-58

Rabindran SK, Giorgi G, Clos J, Wu C (1991) Molecular cloning and expression of a human heat shock factor, HSF1. *Proceedings of the National of Academy of Sciences USA* **88**, 6906-6910

Reddy RK, Kurek I, Silverstein AM, Chinkers M, Breiman A, Krishna P (1998) High molecular weight FK506-binding proteins are components of heat shock protein 90 heterocomplexes in wheat germ lysate. *Plant Physiology* **118**, 1395-1401

Richter K, Buchner J (2001) HSP90: Chaperoning signal transduction. *Journal of Cell Physiology* **188**, 281-290

Sangwan V, Dhindsa RS (2002) *In vivo* and *in vitro* activation of temperature-responsive plant MAP Kinases. *FEBS Letters* **531**, 561-564

Sangwan V, Orvar B J, Beyerly J, Hirt H, Dhindsa RS (2002) Opposite changes in membrane fluidity mimic cold and heat shock activation of distinct plant MAP Kinase pathways. *The Plant Journal* **31**, 629-638

Scharf K-D, Siddique M, Vierling E (2001) The expanding family of *Arabidopsis thaliana* small heat shock proteins and a new family of proteins containing α-crystallin domains (Acd proteins). *Cell Stress Chaperones* **6**, 225-237

Schöffl F, Prändl R, Reindl A (1998) Regulation of the heat-shock response. *Plant Physiology* **117**, 1135-1141

Schuetz TJ, Gallo GJ, Sheldon L, Tempst P, Kingston RE (1991) Isolation of a cDNA for HSF2: evidence for two heat shock factor genes in humans. *Proceedings of the National of Academy of Sciences USA* **88**, 6911-6915

Stancato LF, Hutchison KA, Krishna P, Pratt WB (1996) Animal and plant cell lysates share a conserved chaperone system that assembles the glucocorticoid receptor into a functional heterocomplex with hsp90. *Biochemistry* **35**, 554-561

Sorger PK (1991) Heat shock factor and the heat shock response. *Cell* **65**, 363-366

Suri SS, Dhindsa RS (2008) A heat-activated MAP kinase (HAMK) as a mediator of heat shock response in tobacco cells. *Plant, Cell and Environment* **31**, 218-226

Takaki E, Fujimoto M, Nakahari T, Yonemura S, Miyata Y, Hayashida N, Yamamoto K, Vallee RB, Mikuriya T, Sugahara K, Yamashita H, Inouye S, Nakai A (2007) Heat shock transcription factor 1 is required for maintenance of ciliary beating in mice. *The Journal Biological Chemistry* **282**,

37285-37292

Towill LE, Mazur P (1974) Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures: *Canadian Journal of Botany* **53**, 1097-1102

Waters ER, Lee GJ, Vierling E (1996) Evolution, structure and function o the small heat shock proteins in plants. *Journal of Experimental Botany* **47**, 325-338

Wu C (1995) Heat shock transcription factors. *Annual Review of Cell Biology* **11**, 441-469

Yokotani N, Ichikawa T, Kondou Y, Matsui M, Hirochika H, Iwabuchi M, Oda K (2007) Expression of rice heat shock transcription factor OsHsfA2e enhances tolerance to environmental stresses in transgenic *Arabidopsis*. *Planta* **227**, 1432-2048

Zhang Z, Quick MK, Kanelakis KC, Gijzen M, Krishna P (2003) Characterization of a plant homolog of Hop, a cochaperone of Hsp90. *Plant Physiology* **131**, 525-535

Zhang S, Klessig D (2001) MAPK cascades in plant defense signaling. *Trends in Plant Science* **6**, 520-527

Zwerger K, Hirt H (2001) Recent advances in plant MAPK kinase signalling. *The Journal of Biological Chemistry* **382**, 1123-1131