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Ca^{2+} and Calmodulin Modulate DNA-Binding Activity of Maize Heat Shock Transcription Factor in Vitro

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DNA-binding activity of a maize heat shock transcription factor (HSF) was induced by heat shock of a whole cell extract at 44°C. Addition of the calcium ion chelator EGTA reduced the binding of the HSF to heat shock element (HSE) in vitro. Re-addition of $CaCl_2$ to the sample pretreated with EGTA restored the ability of the HSF to bind to DNA. DNA-binding activity of the HSF was also induced by directly adding $CaCl_2$ to a whole cell extract at non-heat-shock temperature, but not by $MgCl_2$. During HS at 44°C, calmodulin (CaM) antagonists chlorpromazine (CPZ) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) inhibited DNA-binding activity of the HSF in a concentration-dependent manner, but *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5), an inactive structural analogue of W7, did not. Addition of antiserum specific to CaM reduced the binding of the HSF to HSE. Re-addition of CaM to the sample pretreated with antiserum could restore the binding activity of the HSF. DNA-binding activity of the HSF was promoted by directly adding CaM to a whole cell extract at 44°C, but not by BSA. Moreover, at non-heat-shock temperature, DNA-binding activity of the HSF was also induced by directly adding CaM to a whole cell extract, but not by BSA. Our observations further confirm the role of Ca^{2+} in activation of the HSF in plant and provide the first example of the role of CaM in regulation of DNA-binding activity of the HSF. These results suggest that Ca^{2+} and CaM are involved in HSP gene expression likely through regulating the activity of the HSF.

Keywords: Ca^{2+} — Calmodulin — DNA-binding activity — Heat shock transcription factor — Maize.

Abbreviations: CaM, calmodulin; CPZ, chlorpromazine; DTT, dithioreitol; HS, heat shock; HSE, heat shock element; HSF, heat shock transcription factor; HSPs, heat shock proteins; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide.

Introduction

All organisms respond to elevated temperatures and many other stresses by production of a defined set of proteins called heat shock proteins (HSPs). In eukaryotes, the expression of the HSP genes encoding the different HSPs is mediated by heat shock transcription factors (HSFs). They are the terminal components of a signal transduction chain mediating the activation of genes responsive to both heat stress and a large number of chemical stressors. Sequencing analysis of *Arabidopsis* genome revealed a unique complexity of the plant HSF family (Nover et al. 2001). The regulation of activity of the HSF is a central mechanism of transcriptional regulation for HSP gene expression. Under normal growth condition, the HSF is maintained in an inert monomer state through association with molecular chaperones such as HSP70 (Mosser et al. 1993, Shi et al. 1998). During heat shock (HS), the HSF is converted from a transcriptional inactive monomer to active trimmer that is capable of binding to conservative promoter elements (heat shock elements, HSEs) and exhibits transcriptional activity (Craig et al. 1994, Wu 1995). The importance of HSEs for heat-dependent transcriptional regulation in plant has been verified in transgenic tobacco. They exist as multiple copies in promoter region (Gurley and Key 1991, Shimizu et al. 1996, Schöffl et al. 1998). Schöffl described the basic structure of the HSE for transcriptional regulation of eukaryotic HSP genes. It represents a palindrome element with repetitive purine and pyrimidine motifs: 5′-nGAAnnTTCnnGAAnnTTCn-3′ (Schöffl et al. 1998). In addition, the activity of the HSF is also influence by phosphorylation (Reindl et al. 1997, Morimoto 1998, Chu et al. 1998, Xia et al. 1998, Holmberg et al. 2001). Some biochemical and genetic evidence also suggested a role of HSPs in the negative regulation of HS response (Lee and Schöffl 1996, Zou et al. 1998, Bharadwaj et al. 1999, Bonner et al. 2000, Kim and Schöffl 2002).

Elevated temperature initiates changes in transcription and selective translation of mRNA encoding HSPs, thereby enhancing thermotolerance of the treated plants. In the plant kingdom especially, however, the pathways by which HS signals are perceived and transduced to activate HSP gene expression and induce thermotolerance are less understood.

The second-messenger Ca^{2+} was found to be involved in regulation of many responses of plants to environmental signals. Ca^{2+} level often shows significant changes in plant cells under the influence of various stress signals such as cold shock or mechanical stimulation (Knight et al. 1991, Knight et al. 1992). Calmodulin (CaM) is a multifunctional receptor for

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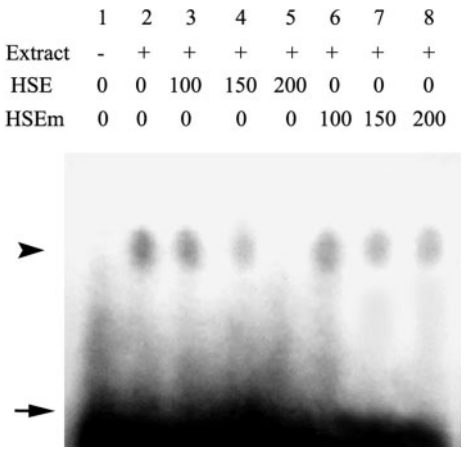

Fig. 1 Specificity of binding of HSF to synthetic HSE in vitro induced by HS at 44°C. Twenty μl of reaction mixtures containing 1 ng of labeled HSE in binding buffer were incubated at 20°C for 15 min in the presence (lanes from 2 to 8) or absence (lane 1) of maize whole cell extract (10 μg of protein) after HS treatment at 44°C for 30 min. HS treatment induced formation of HSE-specific complex (lane 2) that was indicated by an arrowhead. The addition of 100-fold excess unlabeled HSE to the reaction mixture slightly decreased the formation of HSE-specific complex (lane 3). One hundred and fifty-fold excess of the unlabeled HSE had a distinct competitive effect (lane 4). The HSE-specific complex disappeared in the presence of 200-fold excess unlabeled HSE (lane 5), but not of the same concentration of mutant HSE (lanes 6–8). The reaction mixture assayed in lane 1 did not contain extract (–). Free HSE oligonucleotide is indicated by an arrow.

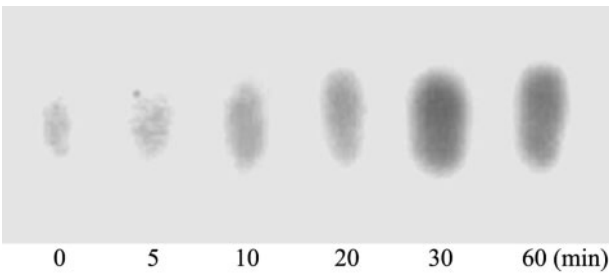

Fig. 2 The kinetics of binding of HSF to HSE in vitro. Gel mobility shift analysis was used to assay DNA-binding activity of HSF in whole cell extracts which were heat-shocked at 44°C for 0–60 min as indicated below each lane.

intracellular Ca^{2+} signal. It regulates a number of intracellular physiological processes. In recent years it has been reported that intracellular Ca^{2+} level is significantly elevated during HS (Gong et al. 1998, Larkindale and Knight 2002). The increase in Ca^{2+} level results in promoting HSP synthesis induced by HS (Trofimova et al. 1999). HS also up-regulates the level of CaM protein in maize seedlings (Gong et al. 1997). Expression of the calmodulin-related touch (TCH) genes in heat shocked *Arabidopsis thaliana* cells has also been observed (Braam 1992). The role played by Ca^{2+}-CaM signal system in regulation of HSP gene expression has recently been studied in our laboratory. Our previous work shows that there is a CaM-binding site within maize cytoplasm HSP70 and that HSP70 binds CaM in a Ca^{2+}-dependent manner (Sun et al. 2000). Our observations with wheat indicate that intracellular Ca^{2+} and CaM levels increase during HS; Ca^{2+} induces a strongly up-regulated expression of CaM gene; Ca^{2+} and CaM all promote the expression of HSP genes and synthesis of HSPs (Fan et al. 2000, Liu et al. 2003).

It was reported that in vitro DNA-binding activity of the HSF was promoted by Ca^{2+} in HeLa cells (Mosser et al. 1990), but correlative experiments in higher plants have not been reported. The involvement of CaM in regulating DNA-binding activity of the HSF has not been documented in either animal or plant cells. We report here the activation of HSE-binding ability of maize HSF in vitro by Ca^{2+} and CaM, and suggest that the Ca^{2+}-CaM signal system is involved in the regulation of HSP gene expression, probably through promoting DNA-binding activity of the HSF.

Results

Activation of HSF by HS in vitro

Similar to results obtained with other eukaryotic organisms (Mosser et al. 1990, Zimarino et al. 1990, Scharf et al. 1990, Hübel and Schöffl 1994, Czarnecka et al. 1995), induction of HSP gene in maize is associated with the activation of a preformed HSE-binding protein. Fig. 1 shows gel mobility shift analysis of a heat-shocked extract from maize. A synthetic double-stranded oligonucleotide containing the optimal HSE core consensus was used as a probe. Heat shock treatment (44°C, 30 min) induced formation of HSE–protein complex (Fig. 1, lane 2, shown by arrowhead). The complex was separated from free radiolabeled HSE oligonucleotide by electrophoresis on native polyacrylamide gels. The free radiolabeled HSE oligonucleotide migrated to the bottom of the gel, while the HSE–protein complex migrated more slowly. To demonstrate specificity of the binding of the protein to HSE in vitro, we performed a competitive binding experiment. The formation of HSE–protein complex decreased slightly in the presence of 100-fold excess unlabeled HSE oligonucleotide in the binding reaction mixture with a heat-shocked whole cell extract (Fig. 1, lane 3). Complex formation with the labeled HSE oligonucleotide was efficiently competed by 150-fold excess of the unlabeled HSE oligonucleotide (Fig. 1, lane 4). Two hundred-fold excess of the unlabeled HSE oligonucleotide completely competed with the binding role of HSE to the protein (Fig. 1, lane 5). The same concentration of mutant HSE (HSEm) in which some essential bases for specific binding were replaced by other bases did not have the competitive effect (Fig. 1, lanes 6, 7, 8). The results indicate that the binding of the protein to HSE is specific. HSE is a specific binding site for HSF, and the sequence specificity of the synthetic HSE oligonucleotide-HSF

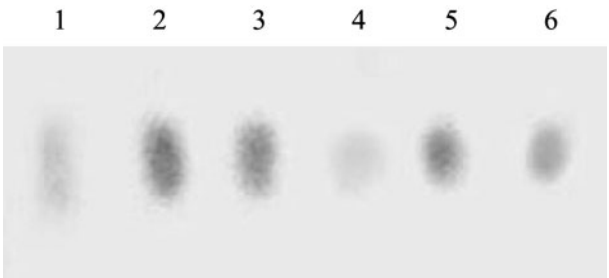

Fig. 3 The effects of EGTA and re-addition of $CaCl_2$ after pretreatment with EGTA on DNA-binding activity of the HSF at 44°C. A whole cell extract was treated with EGTA or $CaCl_2$ at 44°C for 30 min. Lane 1, treatment with distilled water at 27°C as control; lane 2, treatment with distilled water at 44°C; lane 3, treatment with 1 mM EGTA at 44°C; lane 4, treatment with 5 mM EGAT at 44°C; lane 5, treatment with 5 mM EGTA prior to 1 mM $CaCl_2$ at 44°C; lane 6, treatment with 5 mM EGTA prior to 5 mM $CaCl_2$ at 44°C.

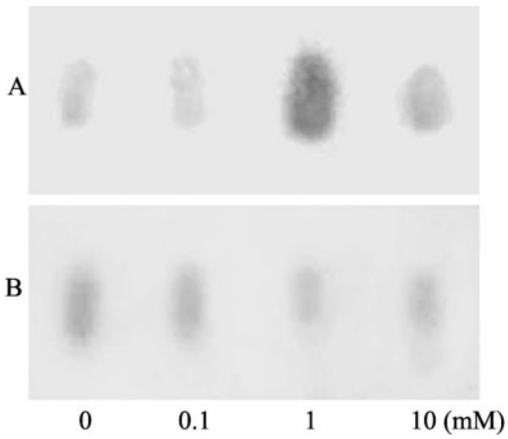

Fig. 4 The effects of $CaCl_2$ on the DNA-binding activity of HSF at non-HS temperature. A whole cell extract was incubated at 27°C for 1 h with various concentrations of $CaCl_2$ (A), as indicated below each lane. $MgCl_2$ was used as control (B).

interaction was well documented in tomato, soybean and *Arabidopsis* (Scharf et al. 1990, Czarnecka et al. 1995, Hübel and Schöffl 1994), thus the HSE-binding protein is indeed HSF.

The appearance of in vitro activated HSF was examined by performing a gel mobility shift assay (Fig. 2). Samples of whole cell extract were heat-shocked in a water bath at 44°C for 0, 5, 10, 20, 30 and 60 min. Little HSE-binding activity of HSF was observed in whole cell extract at the beginning of HS at 44°C. The level of HSE–HSF complexes increased gradually with time of HS, beginning 5 min after onset of HS treatment. A significant increase was observed in 20 min of HS treatment. The level of binding of HSF to HSE reached its maximum after 30 min of HS. DNA-binding activity of the HSF increased in a time-dependent manner.

Activation of HSF by Ca^{2+} in vitro

Some reagents, such as $CaCl_2$, $MgCl_2$ and the Ca^{2+} chelator EGTA, were employed to investigate the role of Ca^{2+} in activation of HSF. DNA-binding activity of the HSF was induced by HS treatment at 44°C for 30 min (Fig. 3, lane 2) as compared with the control which was treated at 27°C (Fig. 3, lane 1). Under HS at 44°C, addition of EGTA to the whole cell extract affected the binding of the HSF to HSE, 1 mM EGTA lowered the activity of HSF weakly, while 5 mM EGTA decreased activity of HSF significantly (Fig. 3, lanes 3, 4). Addition of 1 mM or 5 mM $CaCl_2$ to the extract pretreated by 5 mM EGTA partly restored the binding activity of the HSF to HSE (Fig. 3, lanes 5, 6). The effect of 1 mM $CaCl_2$ was more obvious than that of 5 mM $CaCl_2$. The results above indicate that Ca^{2+} is involved in the activation of HSF induced by HS, and the effect is specific to Ca^{2+}. They also indicate that optimal concentration of Ca^{2+} needed in signal transduction is relatively low. We consider that excessive high concentration of Ca^{2+} might affect metabolic processes.

In addition, DNA-binding activity of the HSF was induced by directly adding $CaCl_2$ to a whole cell extract at non-HS temperature (27°C, Fig. 4A), but not by $MgCl_2$ (Fig. 4B). Addition of 1 mM $CaCl_2$ or 10 mM $CaCl_2$ increased activity of HSF. The effect of 1 mM $CaCl_2$ was most prominent. The same concentration of $MgCl_2$ (as control) had no promoting effect on the activity of the HSF. The observation indicates that at non-HS temperatures, Ca^{2+} can activate DNA-binding activity of the HSF in the place of HS treatment.

Activation of HSF by CaM in vitro

The effects of CaM antagonists on DNA-binding activity of HSF at 44°C—To examine the involvement of CaM in DNA-binding activity of the HSF, we treated whole cell extract of maize seedlings with CaM antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), at 44°C for 30 min (Fig. 5A). Addition of 100 μM W7 had little effect on activity of the HSF. The activity of the HSF was inhibited intensely by 150 μM and 200 μM W7, but not by *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5), an inactive structural analogue of W7 (Fig. 5B). Addition of another CaM antagonist, chlorpromazine (CPZ), (from 25 μM to 100 μM) also decreased the binding of HSF to HSE in a concentration-dependent manner. The extent of inhibition increased gradually with increasing concentration (Fig. 5C).

The effects of CaM antiserum on the DNA-binding activity of the HSF at 44°C—To further confirm the effect of CaM on the DNA-binding activity of the HSF, we treated whole cell extract of maize seedlings with antiserum specific to CaM, at 44°C for 30 min (Fig. 6). Addition of antiserum specific to CaM to the whole cell extract reduced the binding of the HSF to HSE. The extent of inhibition was dependent on amount of CaM antiserum. Addition of 1 : 200 dilution or 1 : 100 dilution of CaM antiserum caused a remarkable decrease in HSE-binding activity of the HSF (Fig. 6, lanes 3, 4), while high concentration of antiserum (1 : 50 dilution) almost completely inhibited the binding of HSF to HSE (Fig. 6, lane 5). Re-

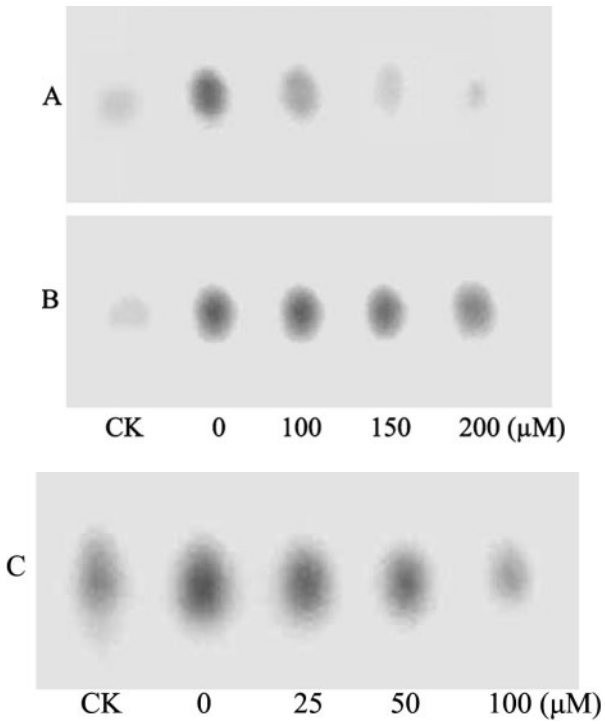

Fig. 5 The effects of CaM antagonists on the DNA-binding activity of HSF at 44°C. A whole cell extract was heat shocked at 44°C for 30 min with various concentrations of W7 (A) or CPZ (C), as indicated below each lane. W5, an inactive structural analogue of W7, was used as a control (B). Lane CK, an untreated whole cell extract incubated at 27°C.

addition of 10^{-8} M or 10^{-7} M CaM to the sample pretreated with 1 : 50 antiserum partly restored the ability of the HSF to bind to HSE (Fig. 6, lanes 6, 7). The effect of 10^{-7} M CaM was more substantial. The results suggest that CaM could be involved in the activation of the HSF induced by HS.

The effects of purified CaM on DNA-binding activity of HSF at 44°C—DNA-binding activity of the HSF could be induced by directly adding CaM to a whole cell extract at 44°C. The whole cell extract pretreated with CaM or bovine serum albumin (BSA) was heat-shocked for 30 min at 44°C before gel mobility shift assay was performed (Fig. 7). DNA-binding activity of the HSF in the cell extract without addition of CaM is relatively low. Addition of 10^{-9}, 10^{-8} M BSA (control) had a slightly promoting effect. It was possible that BSA was partly denatured by HS treatment at 44°C for 30 min. The activation of the HSF induced by denatured proteins under HS had been documented (Zou et al. 1998); therefore BSA here was likely to play the role as denatured protein. However, CaM is a heat-resistant protein in comparison with BSA and the same concentration of CaM distinctly promoted the activity of the HSF for binding to HSE, indicating that DNA-binding activity of the HSF might be also promoted by CaM besides by denatured protein.

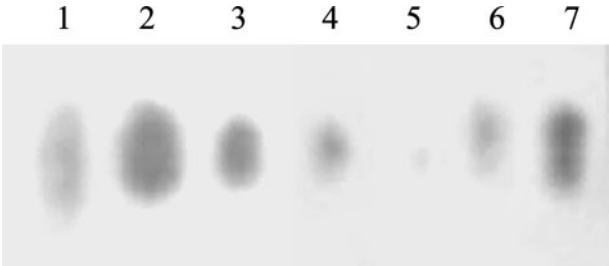

Fig. 6 The effects of antiserum specific to CaM, and re-addition CaM on the DNA-binding activity of HSF at 44°C. A whole cell extract was treated with CaM antiserum or CaM at 44°C for 30 min. Lane 1, treatment with distilled water at 27°C as control; lane 2, treatment with distilled water at 44°C; lane 3, treatment with 1 : 200 dilution of CaM antiserum at 44°C; lane 4, treatment with 1 : 100 CaM antiserum at 44°C; lane 5, treatment with 1 : 50 CaM antiserum at 44°C; lane 6, treatment with 1 : 50 CaM antiserum followed by 10^{-8} M CaM at 44°C; lane 7, treatment with 1 : 50 CaM antiserum followed by 10^{-7} M CaM at 44°C

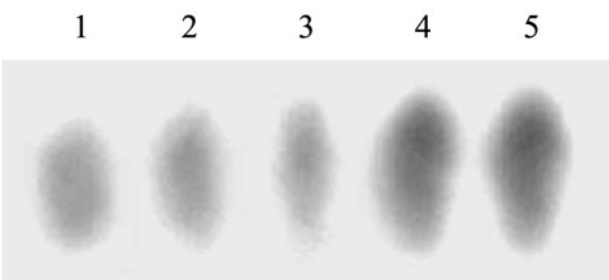

Fig. 7 The effects of CaM on the DNA-binding activity of HSF at 44°C. A whole cell extract pretreated with CaM or BSA was heat-shocked at 44°C for 30 min before gel mobility shift assay was performed. Lane 1, treatment with 10^{-8} M BSA; lane 2, treatment with 10^{-9} M BSA; lane 3, treatment with distilled water; lane 4, treatment with 10^{-9} M CaM; lane 5, treatment with 10^{-8} M CaM.

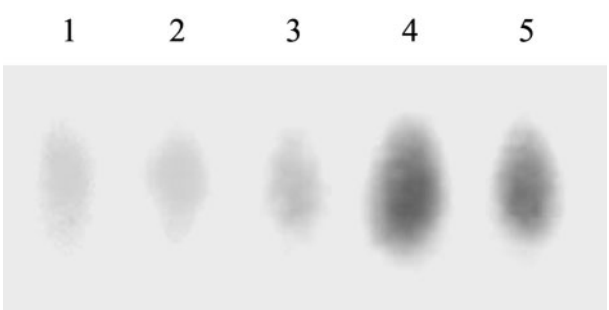

Fig. 8 The effects of CaM on the DNA-binding activity of HSF at non-HS temperature. A whole cell extract pretreated with CaM or BSA was incubated at 27°C for 1 h before gel mobility shift assay was performed. Lane 1, treatment with 10^{-8} M BSA; lane 2, treatment with 10^{-9} M BSA; lane 3, treatment with distilled water; lane 4, treatment with 10^{-9} M CaM; lane 5, treatment with 10^{-8} M CaM.

The effects of purified CaM on DNA-binding activity of HSF at non-HS temperature—DNA-binding activity of the HSF might also be induced by directly adding CaM to a whole

cell extract at non-HS temperature (27°C). CaM or BSA was added directly to non-heat-shocked whole cell extract before a gel mobility shift assay was performed. DNA-binding activity of the HSF in the cell extract without addition of CaM is very low, addition of 10^{-9}, 10^{-8} M CaM clearly promoted activity of HSF for binding to HSE, but the same concentration of BSA (control) did not (Fig. 8). The observations indicate that at non-HS temperature CaM may activate DNA-binding activity of the HSF instead of HS treatment.

The promoting role of 10^{-8} M CaM was weaker than that of 10^{-9} M CaM under non-HS, but was not so under HS at 44°C. It is probably because there is distinct metabolic action under HS and non-HS. In addition, control experiments showed that at 27°C treatment with antiserum against CaM, EGTA, W7 or CPZ had little inhibitory effects on basal activity of HSF (data not shown).

Discussion

Studies of HS have brought new insights into essential cellular processes including the transduction of external signals through cell membranes and cytoplasm to the nucleus. How HS signals are transduced to activate HSP genes is presently of intense interest. It was shown that many signal transduction components in cells such as Ca^{2+} and CaM are altered prior to the expression of HSP genes. A significant change in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) induced by HS was reported in both animal (Calderwood et al. 1988) and plant cells (Biyaseheva et al. 1993, Gong et al. 1998, Liu et al. 2003). The accumulation of CaM protein induced by HS was observed in maize seedlings (Gong et al. 1997). We also reported that HS induced the expression of CaM gene *CaM1-2* and the accumulation of the CaM protein in wheat seedlings (Fan et al. 2000, Liu et al. 2003). The roles of Ca^{2+}-CaM in regulation of HSP gene expression and synthesis of HSPs were observed recently in our laboratory. The expression of *hsp26* and *hsp70* genes was up-regulated by addition of $CaCl_2$, and down-regulated by EGTA, the calcium ion channel blockers $LaCl_3$ and verapamil, or the calmodulin antagonists W7 and CPZ. Treatment with $CaCl_2$ also increased, and with EGTA, verapamil, CPZ or TFP decreased, synthesis of HSPs. Additionally, the expression of wheat *CaM1-2* gene was up-regulated only 10 min after HS, later than the increase in $[Ca^{2+}]_i$ but earlier than HSP gene expression (Liu et al. 2003). All of results above indicate that Ca^{2+}-CaM is directly involved in the HS signal transduction pathway. But the mechanism by which Ca^{2+}-CaM regulates the HSP gene expression is still unknown.

The transcriptional activation of HSP genes is mediated by a pre-existing HSF. A major goal to understand the regulation of HS-inducible transcription is the identification of the intracellular signals that activate HSF. Thus we mainly focused on the relationship between the Ca^{2+}-CaM signal system and HSF activity.

Herein, we report that Ca^{2+}-CaM is involved in the binding activity of maize HSF to HSE in vitro by the gel mobility shift assay with γ-^{32}P-labeled HSE as the probe. Competitive binding experiments indicate that the binding of the HSF to synthetic HSE is specific (Fig. 1). The binding activity of the HSF to HSE was induced by HS (44°C) of a maize whole cell extract. DNA-binding activity of the HSF increased in a time-dependent manner, it increased gradually with time of HS and reached its maximum after 30 min of HS (Fig. 2). Then we further studied the roles of Ca^{2+} and CaM in the regulation of the activity of the HSF. It was reported that DNA-binding activity of the HSF was promoted by Ca^{2+} at non-HS temperature in HeLa cells, the promoting effect of 1 mM $CaCl_2$ was the most prominent and 25 mM EGTA reversed the active role (Mosser et al. 1990). Our results here indicated that under non-HS conditions DNA-binding activity of maize HSF was promoted by 1 mM $CaCl_2$, but not by the same concentration of $MgCl_2$ (Fig. 4). In addition, under HS at 44°C, addition of EGTA to whole cell extract of maize seedlings reduced the binding of the HSF to HSE and re-addition of $CaCl_2$ to the sample pretreated by EGTA restored the binding activity of the HSF to HSE (Fig. 3). The results provided the evidence for promoting effect of Ca^{2+} on DNA-binding activity of the HSF in plants. However, direct effect of CaM on DNA-binding activity of the HSF has not ever been investigated previously. Our observations here indicated that DNA-binding activity of the HSF was decreased by treatment with the CaM antagonists W7 and CPZ at 44°C (Fig. 5). Immuno-removal of CaM also lowered DNA-binding activity of the HSF, and subsequent re-addition of purified CaM could restore it at 44°C (Fig. 6). Direct addition of purified CaM to whole cell extract of maize seedlings promoted DNA-binding activity of the HSF at 44°C (Fig. 7). Moreover, at non-HS temperature addition of purified CaM also increased DNA-binding activity of the HSF instead of HS treatment (Fig. 8). The results provide the first evidence for the involvement of CaM in the regulation of the activity of HSF.

A whole cell extract (crude extract) but not a purified recombinant HSF was subjected to HS treatment prior to the binding assay in our experiments. The method with a whole cell extract has some merits (Zou et al. 1998), viz., (1) it recapitulates the initial step of HSF activation in vivo, which is the conversion of inactive, nontrimeric factor to trimmer factor capable of binding HSE, (2) it is capable of sensing heat stress and (3) it is possible that Ca^{2+}-CaM indirectly affect the activity of HSF through some components in the cell (through binding to HSP70, kinase or phosphatase). There are most of the components needed in a whole cell extract. However, a whole cell extract used to HS treatment would not mimic cellular conditions in their entirety. Two important upstream events in HS signal transduction pathway such as a calcium influx and increased CaM expression or activity would fail to occur. Though it is better to do experiments with living seedlings subjected to HS prior to cell free extraction, it is not possible in maize. Initially we tried to do experiments with living seedlings

subjected to HS prior to cell free extraction, but the results showed that the DNA-binding activity of the HSF could not be induced distinctly by HS (data not shown). Perhaps it was because some changes caused by HS were reversed during preparation of maize cell extract. A calcium influx and increased CaM expression after HS have been proven in our previous work (Liu et al. 2003). In this paper we focus our attention on the role of elimination or addition of Ca^{2+} or CaM in the activity of the HSF. The HSE–HSF complex seen in the binding assay might reflect the role of Ca^{2+} or CaM.

Our experimental result (Fig. 2) in this paper showed that HS might activate the HSF in cell-free system without addition of Ca^{2+} and CaM. It is possible that stress-denatured proteins induce the activity of the HSF. Ananthan et al. (1986) proposed that the accumulation of stress-denatured proteins could be the signal for increased expression of HSP genes. However, the observations here also indicate that Ca^{2+} and CaM do contribute to the increase of HS-induced activity of the HSF. In our previous experiments, it was proven that HS mobilized Ca^{2+}-CaM signal system and Ca^{2+} -CaM was involved in the regulation of expression of HSP genes (Liu et al. 2003). Based on our and other evidence, we proposed a Ca^{2+}-CaM pathway of HS signal transduction (Li et al. 2002, Liu et al. 2003). The HS signals are perceived by an as yet unidentified receptor. Receptor activation is closely followed by an increase in $[Ca^{2+}]_i$ through the opening of Ca^{2+} channels in the plasma membrane or intracellular Ca^{2+} store. This elevated level of cytoplasm $[Ca^{2+}]_i$ directly activates CaM and promotes the expression and accumulation of CaM. Activated CaM promotes the activity of HSF. Then activation of HSF initiates the transcription and translation of HSP genes. The proposal is supported by the results in this paper. However, the mechanism by which the activity of the HSF is regulated by CaM remains unclear. Holmberg et al. (2001) reported the increase of level of in vivo Ser230 phosphorylation and transactivation of HSF caused by Ca^{2+}/CaM-dependent protein kinase II (CaMKII). Our recent observation showed that cyclosporin A, a specific inhibitor of calcineurin (Ca^{2+}/CaM-dependent protein phosphatase), promoted the expression of *hsp18.6* gene in transgenic *Arabidopsis* (not published). The results indicate that CaM regulates the activity of HSF probably through regulating HSF phosphorylation by CaM-dependent kinase or phosphatase. Phosphorylation of HSF can be regulated by distinct protein kinases/phosphatases at multiple phosphorylation sites and is a dynamic process (Chu et al. 1996, Kline and Morimoto 1997, Holmberg et al. 2001). Another possible mechanism for the activation of HSF by CaM is through HSP70 pathways. There is a CaM-binding site within HSP70 (Stevenson and Calderwood 1990, Sun et al. 2000). Thus CaM can directly interact with HSP70, causing the HSP70–HSF complex to release and activate HSF. The exact mechanism by which the activity of the HSF is regulated by Ca^{2+}-CaM remains to be confirmed and is under study in our laboratory. Besides the roles of the stress-denatured proteins and Ca^{2+}-CaM, there are probably other regulative factors for HSF activity (Mosser et al. 1990, Sung et al. 2003). The multiple regulatory pathways are likely to crosstalk or cooperate with each other to provide a precise and elegant mechanism.

Materials and Methods

Plant materials and growth conditions

Maize (*Zea mays* L.), inbred line H21, seeds were obtained from Hebei Academy of Agricultural Sciences, P.R. China. Seeds were imbibed overnight (15 h) in distilled water at 27°C. Swelled seeds were sown in glass dishes spread with six layers of filter paper wetted with distilled water, for germination at 27°C in the dark for 4 d. Four-day-old etiolated seedlings were used in all experiments.

Oligonucleotides and labeling

Heat shock element and mutant HSE (HSEm) were synthesized by sbs Corporation, Beijing, P.R. China. The sequences of HSE and HSEm are from Scharf (Scharf et al. 1990) and Hübel (Hübel and Schöffl 1994). For the gel mobility shift assay the two HSE oligonucleotides were annealed and labeled by incubation with [γ-^{32}P]ATP using T4 polynuleotide kinase according to an established method (Sambrook et al. 1989).

HSE:

a. 5′ tcgaggatcctaGAAgcTTCcaGAAgcTTCtaGAAgcagatc 3′

b. 3′ cctaggat CTTcgAAGgt CTTcgAAGatCTTcg tctagagct 5′

HSE_m:

a. 5′ tcgaggatcctatAAgcTTacatAAgcTTatatAAgcagatc 3′

b. 3′ cctaggataTTcgAAtgtaTTcgAAtataTTcgtc tagagct 5′

Preparation of CaM and antiserum specific to CaM

The plasmid encoding the potato CaM gene *PCM6* (Takezawa et al. 1995) was obtained from Professor B.W. Poovaiah, Laboratory of Plant Molecular Biology and Physiology, Department of Horticulture, Washington State University, U.S.A. Recombinant potato CaM was purified to electrophoresis homogeneity from *E. coli* using a phenyl-Sepharose affinity column as described (Biro et al. 1984). The antiserum against CaM was prepared and characterized as described (Bai et al. 2002).

Preparation of whole cell extract of maize seedlings and HS treatments

Samples of 0.5 g from 4-day-old etiolated maize seedlings were collected and frozen in liquid nitrogen. After fully grinding, the powder was homogenized in 1 ml ice-cold extraction buffer containing 10 mM HEPES, pH 7.9, 400 mM NaCl, 0.5 mM dithioreitol (DTT), 0.1 mM glycol-*bis*-(α-aminoethyl) ether *N*,*N*,*N*,*N*-tetraacetic acid (EGTA), 0.5 mM phenylmethylsulfonyl fluoride and 5% (v/v) glycerol. Homogenates were filtered, then centrifuged at 27000×*g* for 20 min at 4°C. Supernatants were used as the whole cell extract (1,000 μg ml^{-1}). Protein was quantitated by the method of Bradford. For HS treatments, the whole cell extract was placed into a water bath at 44°C for different times, as specified in the various experiments.

Gel mobility shift assay

The binding assay and electrophoresis were carried out as described (Scharf et al. 1990, Czarnecka et al. 1995). One ng of labeled HSE was mixed with a sample of whole cell extract containing 10 μg of protein in 20 μl of binding buffer [20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 15 mM $MgCl_2$, 0.5 mM ethylenediamine tetraacetic acid, 1 mM DTT and 10% (v/v) glycerol]. The mixture was incubated in a water bath at 20°C for 15 min. The protein-bound and free HSE oligonucleotides were eletrophoretically separated on 5% native polyacrylamide gels containing 20% (v/v) glycerol and 1× Tris-Boric acid

buffer. Electrophoresis was performed at 200 V and 20°C for 3 h, followed by autoradiography. All of the experiments were repeated more than three times with reproducible results.

Chemicals

W7, W5 and CPZ were purchased from Sigma, St. Louis, MO, U.S.A. [γ-^{32}P]ATP was from Yahui Bio-medical Technology company, Beijing, P.R. China. All other reagents used was of analytical purity.

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