Calmodulin Is Involved in Heat Shock Signal Transduction in Wheat[1]

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The involvement of calcium and calcium-activated calmodulin (Ca^{2+}-CaM) in heat shock (HS) signal transduction in wheat (*Triticum aestivum*) was investigated. Using Fluo-3/acetoxymethyl esters and laser scanning confocal microscopy, it was found that the increase of intracellular free calcium ion concentration started within 1 min after a 37°C HS. The levels of CaM mRNA and protein increased during HS at 37°C in the presence of Ca^{2+}. The expression of *hsp26* and *hsp70* genes was up-regulated by the addition of $CaCl_2$ and down-regulated by the calcium ion chelator EGTA, the calcium ion channel blockers $LaCl_3$ and verapamil, or the CaM antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and chlorpromazine. Treatment with Ca^{2+} also increased, and with EGTA, verapamil, chlorpromazine, or trifluoperazine decreased, synthesis of HS proteins. The temporal expression of the *CaM1-2* gene and the *hsp26* and *hsp70* genes demonstrated that up-regulation of the *CaM1-2* gene occurred at 10 min after HS at 37°C, whereas that of *hsp26* and *hsp70* appeared at 20 min after HS. A 5-min HS induced expression of *hsp26* after a period of recovery at 22°C after HS at 37°C. Taken together, these results indicate that Ca^{2+}-CaM is directly involved in the HS signal transduction pathway. A working hypothesis about the relationship between upstream and downstream of HS signal transduction is presented.

Organisms have developed a diverse array of mechanisms for adapting to environmental changes. One of the best characterized responses is the induction of heat shock proteins (HSPs). The heat shock (HS) response has been found in almost every organism studied to date. The HSPs are synthesized by cells in response to elevated temperature but are also induced by other environmental stresses (Noven et al., 1992; Kilstrup et al., 1997) and play an important role in the thermotolerance of plants (Queitsch et al., 2000; Burke, 2001). A connection between HS response and oxidative stress has been observed (Gong et al., 1997a; Lee et al., 2000; Larkindale and Knight, 2002; Panchuk et al., 2002). The HSPs are divided into several families based on their molecular mass, and most have molecular chaperones functions (for review, see Boston et al., 1996; Miernyk, 1999). Angiosperms synthesize more small HSPs (smHSPs) than other organisms. These smHSPs are likely critical for survival of heat stress and for specific developmental processes in plants (Waters et al., 1996).

The changes in cytoplasmic calcium levels act as a ubiquitous signal in eukaryotic cells. HS induced a large increase in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) in Chinese hamster (*Cricetulus barabensis*) HA-1 fibroblasts (Calderwood et al., 1988). In plants, Gong et al. (1998) observed that HS caused a transient increase in $[Ca^{2+}]_i$. The change in $[Ca^{2+}]_i$ is also involved in regulating the binding activity of the HS transcription factor (HSF) to the HS element (Mosser et al., 1990), the synthesis of HSPs (Kiang et al., 1994; Kuznetsov et al., 1998), and acquisition of HS-induced thermotolerance in plants (Gong et al., 1997b; Kuznetsov et al., 1998). Calmodulin (CaM) is an important intermediate of calcium-mediated signal transduction. In plants, the role of CaM in regulating a variety of calcium-dependent signaling pathways within the cell (Roberts and Harmon, 1992) and in the extracellular matrix (Ma et al., 1999; Sun et al., 2001) has been documented. The level of CaM protein is also up-regulated by HS in maize (*Zea mays*) seedlings (Gong et al., 1997b), as is expression of CaM-related *TCH* genes in cultured Arabidopsis cells (Braam, 1992).

The downstream events in HS signal transduction have been investigated (for review, see Morimoto, 1998; Schöffl et al., 1998; Pirkkala et al., 2001). In eukaryotes, the expression of the HSP genes induced by HS is mediated by HSF (Wu, 1995; Nover et al., 2001). During HS, the HSF is activated by conversion from a transcriptionally inactive monomer to a trimer. The activation of HSF is also influenced by phosphorylation (Kline and Morimoto, 1997; Reindl et al., 1997). Biochemical and genetic evidence supports a role of HSP70 (and/or HSP90) in negative

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regulation of the HS responses (Lee and Schöffl, 1996; Shi et al., 1998; Zou et al., 1998; Bharadwaj et al., 1999; Bonner et al., 2000; Marchler and Wu, 2001; Kim and Schöffl, 2002).

Although some studies about upstream (primary Ca^{2+}-CaM response) and downstream (expression of HSP genes) of HS signal transduction have been reported, a role of Ca^{2+}-CaM in regulation of HSP gene expression and HSP synthesis has not been documented. Herein, we provide evidence for the involvement of the Ca^{2+}-CaM signaling system in HSP gene expression or HSP synthesis and the order of signal transduction steps during HS. A possible regulatory model of Ca^{2+}-CaM in the signal transduction pathway for heat stress is proposed.

RESULTS

The Increase of $[Ca^{2+}]_i$ during HS of Wheat (*Triticum aestivum*) Cells

To investigate the role of $[Ca^{2+}]_i$ upstream in HS signal transduction, we examined kinetics of change in $[Ca^{2+}]_i$ at the early stage of HS. A thin tissue section was stripped from the sheath of the first leaf of a 10-d-old green wheat seedling and observed using laser scanning confocal microscopy (LSCM). The value of fluorescence intensity is an average value obtained by scanning >10 cells in three different repeats each experiment. The fluorescence intensity did not change (both were 15.3) if 10 μM Fluo-3 in 100 nM $CaCl_2$ solution was observed from 22°C to 37°C (Fig. 1A, 1 and 2), so the effect of temperature on dye fluorescence in the 22°C to 37°C temperature range was negligible. The fluorescence intensity was from 14.3 to 14.6 if the tissue non-loaded was incubated from 22°C to 37°C (Fig. 1A, 3 and 4), showing that autofluorescence during HS was negligible. Treatment with 25 μM A23187 and 5 mM $CaCl_2$ resulted in a fluorescence intensity of 227.2 (Fig. 1A, 6), whereas fluorescence intensity in tissue treated with 5 mM EGTA and 25 μM A23187 was 26 (Fig. 1A, 8). This result verified that Fluo-3-fluorescence increase does report $[Ca^{2+}]_i$ increase. To observe clearly where the dye is located, we made a full LSCM image of a protoplast. The protoplasts were obtained from tissue treated by cellulase and incubated in 10 μM Fluo-3/AM solution at 22°C or 37°C, then observed under LSCM. The image (Fig. 1B) showed that Fluo-3 is located in the cytoplasm. The dye did not move to the vacuole or apoplast during HS treatment. The Fluo-3-fluorescence in the cytoplasm increased obviously during HS treatment (Fig. 1B, 1–4). This proved that HS caused an increase of $[Ca^{2+}]$ in the cytoplasm. In measurement of $[Ca^{2+}]_i$ from wheat tissue during HS, the measured tissue was incubated in medium containing 10 μM Fluo-3/AM at 24°C in the dark for 2 h. Then, the fluorescence of the cells was observed by LSCM. Fluorescence intensity was measured every 0.5 min to a total of 10 min. Control cells maintained at 22°C remained constant in fluorescence during the experiment (Fig. 1, C and E). A significant increase in $[Ca^{2+}]_i$ was observed in the cells during HS at 37°C (Fig. 1, D and E). The initiation of this $[Ca^{2+}]_i$ increase occurred within 1 min of HS. After 4 min of HS, the $[Ca^{2+}]_i$ reached a maximum 3-fold increase (Fig. 1E).

Changes in CaM Protein and mRNA Levels during HS

The levels of CaM protein in tissues treated with distilled water, 10 mM $CaCl_2$, or 5 mM EGTA before HS were similar. The level of CaM protein in tissue with each treatment before HS was normalized to 100%. The concentration of CaM protein in wheat tissue treated with distilled water increased during HS at 37°C and reached a maximum 2-fold increase after 90 min of HS. Treatment with 10 mM $CaCl_2$ promoted the increase during HS at 37°C. The accumulation of CaM protein reached a maximum 3-fold increase after 90 min of HS. The calcium ion chelator EGTA prevented CaM accumulation during HS, suggesting that CaM accumulation is dependent on calcium (Fig. 2).

Northern analysis using the wheat CaM cDNA *CaM1-2* as the probe showed that the *CaM1-2* is constitutively expressed, and its mRNA has a basal expression level at normal temperature (22°C). The *CaM1-2* gene expression started to increase after HS at 37°C for 10 min, then reached its maximum 20 min after HS. The mRNA returned to the basal expression level after 1 h of HS.

The Effect of Exogenous Ca^{2+} on the Expression of Wheat *CaM1-2* and *hsp26* at Non-HS Temperature

The tissue cut from 3-d-old wheat seedlings was incubated in 1-mL solutions of 5, 10, or 50 mM $CaCl_2$, respectively, at 22°C (non-HS temperature) for 30 min. Then, northern analysis using the wheat *CaM1-2* and *hsp26* cDNAs as probes was performed. Wheat *CaM1-2* has a low, basal expression at 22°C (Fig. 3A), and wheat *hsp26* mRNA was undetectable at 22°C (Fig. 3B). Treatment with 5 mM $CaCl_2$ had little effect on the expression of *CaM1-2* and *hsp26*. Treatment with 10 mM $CaCl_2$ promoted expression of the two genes, and the effect of 50 mM $CaCl_2$ was more marked (Fig. 3). Treatment with $MgCl_2$ up to 50 mM did not affect expression of the genes (data not shown).

The Effects of Ca^{2+}-CaM on the Expression of HSP Genes

Various compounds that affect the Ca^{2+}-CaM signaling system were employed to investigate the role of Ca^{2+}-CaM in up-regulating expression of HSP genes. Total RNA was used for northern analysis, using the *hsp26* and *hsp70* cDNAs as probes. In con-

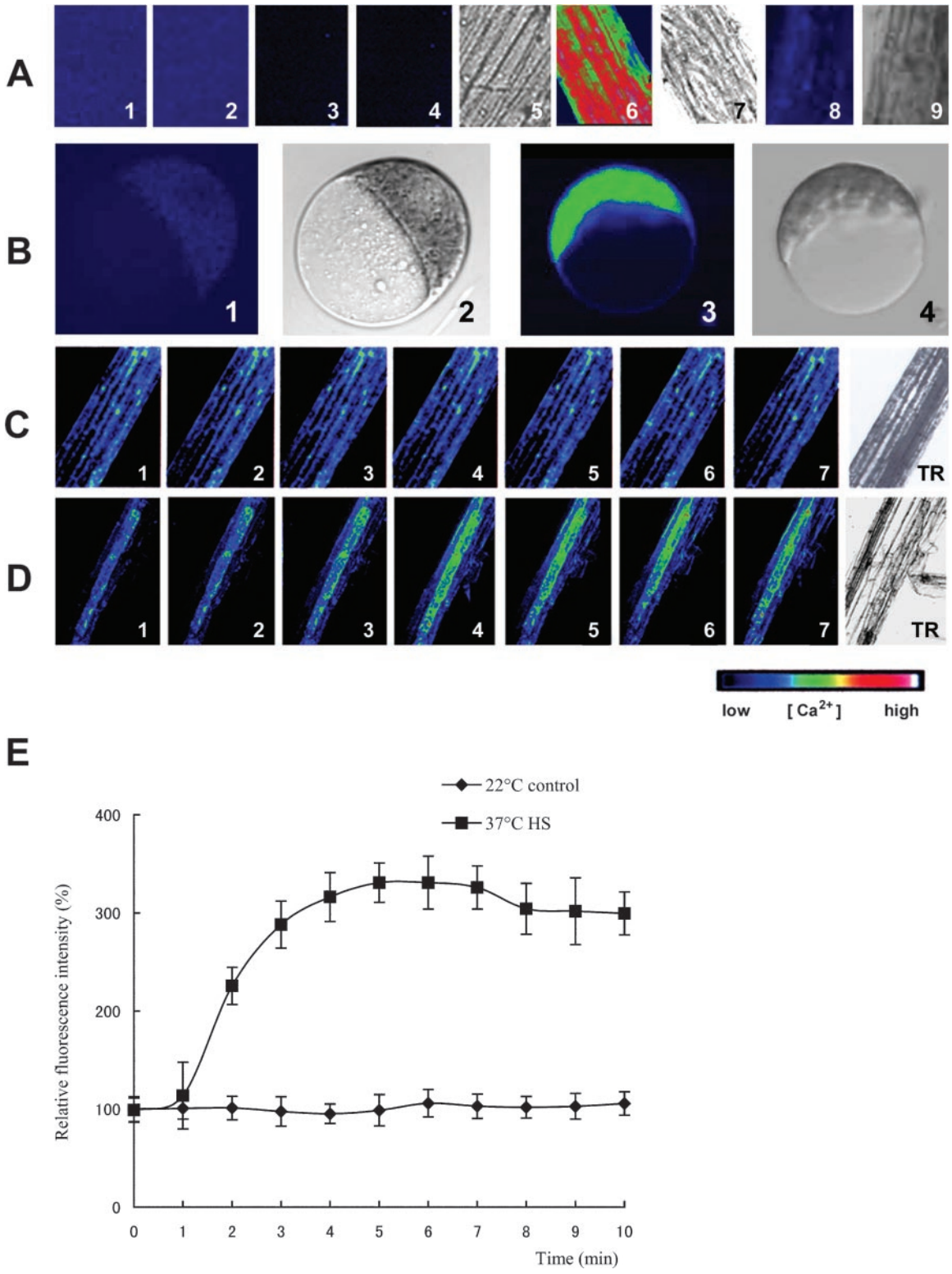

Figure 1. The change in $[Ca^{2+}]_i$ in wheat cells at 22°C or during HS at 37°C. Tissue sections from sheath of first leaf of 10-d-old green wheat seedlings were incubated in medium containing 10 μM Fluo-3/acetoxymethyl esters at 24°C in the dark for 2 h. The tissues loaded with Fluo-3/AM were heat shocked at 37°C or maintained at 22°C as the control and observed by LSCM. Excitation (488 ± 10 nm) and emission (530 ± 40 nm) filters were used in this experiment. The scan mode was XY-T (three dimensional). The fluorescence intensity was measured once every 0.5 min. Fluorescence intensity was an average value of that obtained by scanning over 10 cells from three different repeats of each experiment. A, Lane

(*Legend continues on facing page*)

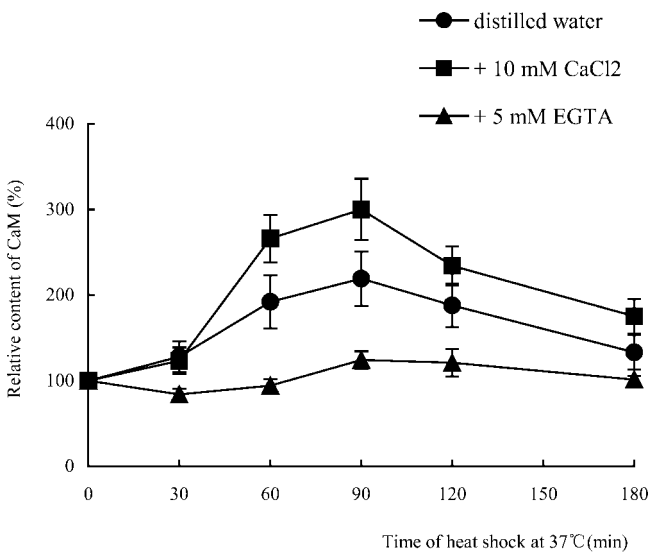

Figure 2. The change in CaM protein level during HS at 37°C. Seeds imbibed overnight were germinated at 22°C in the dark for 3d. The 1.5-cm-long tissues cut from 3-d-old etiolated wheat seedlings were placed wound-side down in 1 mL of 10 mM $CaCl_2$, 5 mM EGTA, or distilled water as a control, and incubated at 37°C. CaM was extracted from treated samples and quantified by ELISA. CaM level in tissue before HS was normalized to 100%. Each data point is the mean of three repeats.

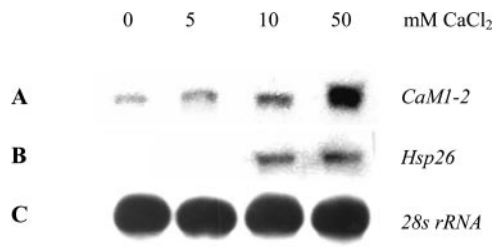

Figure 3. The effects of exogenous Ca^{2+} on the expression of the *CaM1-2* and *hsp26* genes at 22°C. Seedling growth conditions were described in the legend for Figure 2. The tissues were treated with distilled water or 5, 10, or 50 mM $CaCl_2$, respectively, at 22°C for 30 min. Total RNA was extracted and then analyzed by RNA-blot hybridization. Each filter was hybridized with the probe indicated on the right. Equal quantities of total RNA were loaded in each lane.

trol experiments, the treatments with EGTA, $LaCl_3$, or verapamil under non-HS condition (22°C) did not affect expression of *hsp26* or *hsp70*. The *hsp26* mRNA was undetectable, and there was low-level expression of *hsp70* at 22°C (Fig. 4A). A 37°C HS increased the expression of *hsp26* and *hsp70*. Treatment with 10 mM $CaCl_2$ increased the expression of *hsp26* and *hsp70*, whereas treatment with the Ca^{2+} chelator EGTA decreased their expression (Fig. 4B). Treatment with the calcium ion channel blockers showed that 100 μM $LaCl_3$ only slightly lowered the expression of *hsp26*, whereas 300 μM $LaCl_3$ lowered the expression level significantly (Fig. 4C). Expression of *hsp26* was strongly inhibited by both 100 and 200 μM verapamil (Fig. 4D).

The expression of *hsp26* and *hsp70* decreased with increasing concentrations of the CaM antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7). Treatment with 100 and 150 μM W7 had little effect on HSP gene expression, whereas treatment with higher concentrations caused a remarkable decrease in the level of *hsp26* and *hsp70* mRNAs (Fig. 4E). Treatment with 100 to 300 μM *N*-(6-aminohexyl)-1-naphthalene sulfonamide (W5), an inactive structural analog of W7, did not influence the expression of the HSP genes (Fig. 4F). Another CaM antagonist, chlorpromazine (CPZ), also down-regulated the expression of *hsp26* (Fig. 4G). Control experiments showed that at 22°C, treatments with W7 or CPZ did not change the expression of *hsp26* (Fig. 4H).

The Involvement of Ca^{2+}-CaM in Synthesis of HSPs

Proteins were isolated from wheat tissue labeled with [^{35}S]-Met at 37°C or at 22°C (control) and were separated by SDS-PAGE (Fig. 5). At non-HS temperature, the protein patterns from tissue treated with 5 mM EGTA or 50 μM CPZ (data not shown) were similar to that from untreated tissue (Fig. 5, lane 2). In response to HS at 37°C, the synthesis of specific proteins was induced. The proteins with molecular weights corresponding to the 70- to 80-kD HSPs and 17- to 20-kD smHSPs increased during HS (Fig. 5, lane 3). Treatment with 10 mM $CaCl_2$ at 37°C led to increased accumulation of protein bands that correlate mainly with the 70- to 90-kD HSPs and the 26- to

Figure 1. (*Legend continued from facing page.*)
1, dye fluorescence, 10 μM Fluo-3 + 100 nM $CaCl_2$ at 22°C; lane 2, 10 μM Fluo-3 + 100 nM $CaCl_2$ at 37°C; lane 3, autofluorescence in tissue non-loaded dye at 22°C; lane 4, autofluorescence in tissue non-loaded dye at 37°C; lane 5, corresponding bright-field image of cells in lanes 3 and 4; lane 6, Fluo-3-fluorescence in the tissue treated with 5 mM $CaCl_2$ and 25 μM A23187 at 22°C; lane 7, corresponding bright-field image of the cells in lane 6; lane 8, Fluo-3-fluorescence in the tissue treated with 5 mM EGTA and 25 μM A23187 at 22°C; lane 9, corresponding bright-field image of the cells in lane 8. B, Lane 1, Fluo-3-fluorescence in the protoplast at 22°C; lane 2, corresponding bright-field image of protoplast in lane 1; lane 3, Fluo-3-fluorescence in the protoplast at 37°C; lane 4, corresponding bright-field image of protoplast in lane 3. C, Lanes 1 to 7, pseudocolor images of the wheat cells at 22°C for 0, 1.5, 3.0, 4.5, 6.0, 7.5, and 9.0 min, respectively; lane 8, transmission drawing of the cells in lanes 1 to 7 under LSCM. D, Lanes 1 to 7, pseudocolor images of the wheat cells at 0, 1.5, 3.0, 4.5, 6.0, 7.5, and 9.0 min after HS at 37°C; lane 8, the transmission drawing of the cells in lanes 1 to 7 under LSCM. E, The kinetics of $[Ca^{2+}]_i$ in cells from wheat leaf sheath at 22°C or during HS at 37°C. The fluorescent intensity in the figure represents $[Ca^{2+}]_i$.

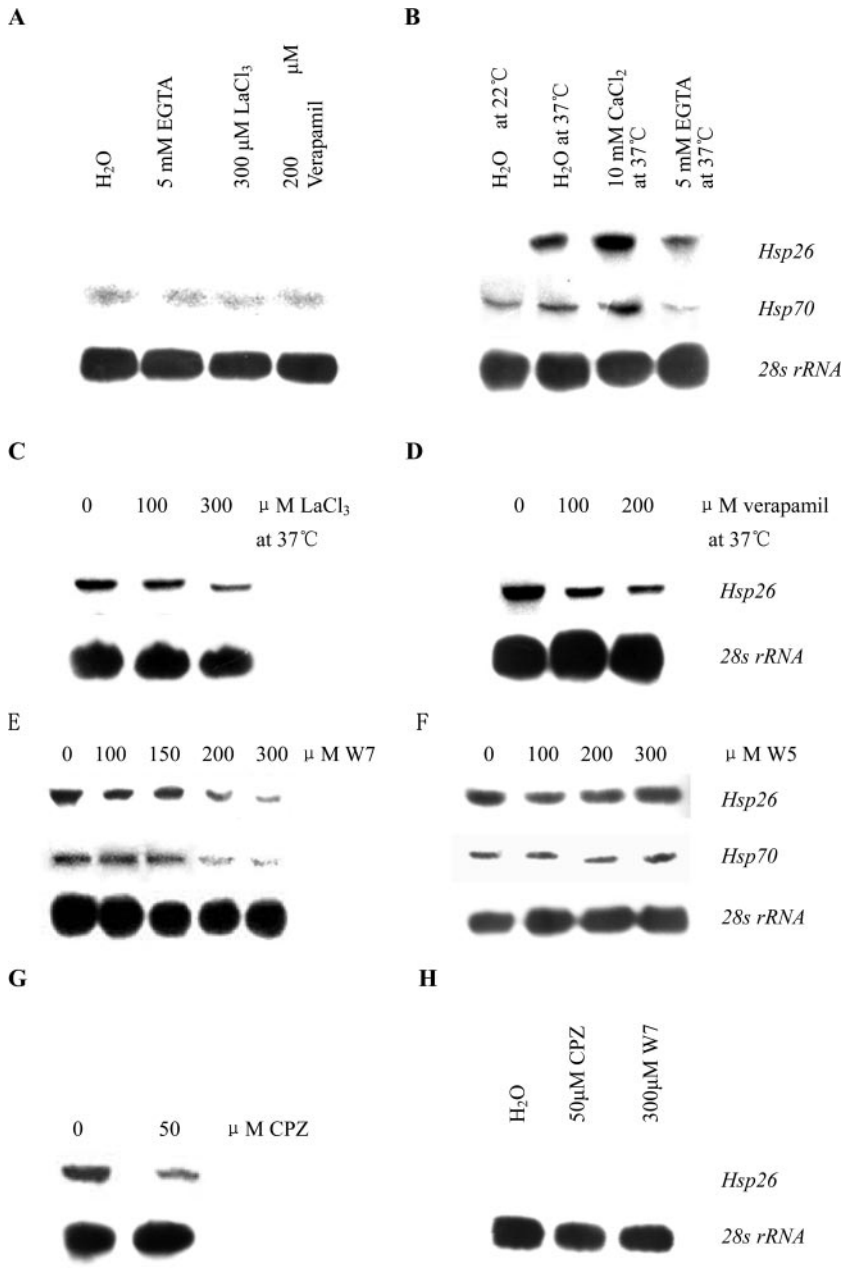

Figure 4. The effects of Ca^{2+}, EGTA, $LaCl_3$, verapamil, and CaM antagonists on the expression of *hsp26* and *hsp70* at 22°C or during HS at 37°C. The growth conditions were the same as described for Figure 2. The tissues were treated with reagents indicated above each lane at 37°C for 1 h (B-G) or kept at 22°C as control (A and H). Total RNA was extracted, followed by northern analysis. Each filter was hybridized with the probe indicated on the right.

28-kD smHSPs (Fig. 5, lane 4). In contrast, when treated with 5 mM EGTA at 37°C, there was decreased synthesis of HS-induced proteins (Fig. 5, lane 5). The synthesis of these proteins induced by HS was also inhibited by treatment with the CaM antagonists, 50 μM CPZ or 25 μM TFP (Fig. 5, lanes 6 and 7).

The Expression Kinetics of CaM1-2 and hsp26 and hsp70 during HS

CaM1-2 has a low, basal level of expression at normal temperature. However, we observed a increase in the accumulation of *CaM1-2* mRNA after treatment at 37°C for only 10 min. The increased level of expression of *CaM1-2* reached its maximum after 20 min of HS, then returned to basal levels after 1 h of HS (Fig. 6A). The *Hsp26* expression was induced by HS treatment. At non-HS temperature, the *hsp26* mRNA was undetectable and was still undetectable 10 min after HS. However, the *hsp26* mRNA levels began to appear 20 min after HS treatment at 37°C. The accumulation of *hsp26* mRNA increased with prolonged HS treatment (Fig. 6B). There was a low, basal level of *hsp70* expression at normal temperature, and expression did not change after HS for 10 min. After HS treatment at 37°C for 20 min, the *hsp70* expression levels began to increase (Fig. 6C). Similar expression patterns for *hsp70* and *hsp26* were observed. The activation of *hsp26* or *hsp70* gene expression was slower than *CaM1-2* expression.

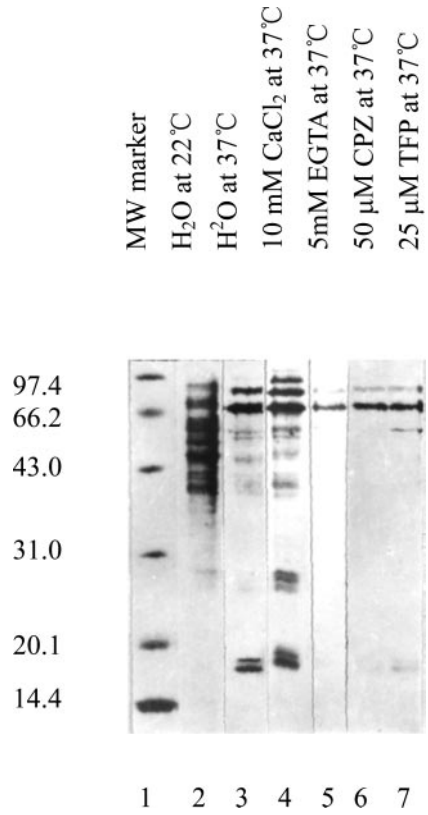

Figure 5. The effects of Ca^{2+}, EGTA, CPZ, or trifluoperazine (TFP) on the synthesis of HSPs at 22°C or during HS at 37°C. The growth conditions were the same as described for Figure 2. The tissues were treated with reagents indicated above each lane at 37°C for 2 h or at 22°C as control. Forty microcuries of [^{35}S]-Met was then added, and labeling was performed for another 2 h at the same temperature. Proteins were isolated from the treated and labeled tissues and separated on an SDS-PAGE. Approximately equal amounts of soluble proteins were loaded. The gels were dried and subjected to autoradiography at −80°C.

To determine the shortest HS treatment that can induce expression of *hsp26*, wheat seedlings were initially heat shocked at 37°C for various lengths of time and then returned to 22°C for recovery. Using this approach, we found that the induction of *hsp26* expression was first detected after only 5 min of HS

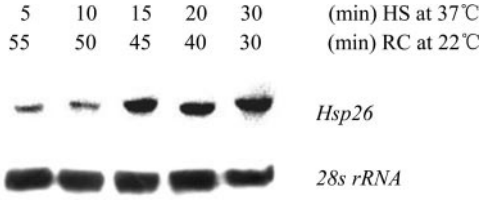

Figure 7. The expression of *hsp26* during a recovery (RC) at 22°C after different HS time at 37°C. The growth conditions were the same as described for Figure 2. The tissues were incubated in distilled water at 37°C and then returned to 22°C for the times indicated above each lane. The total RNA was extracted and analyzed by northern blot. Each filter was hybridized with the probe indicated on the right.

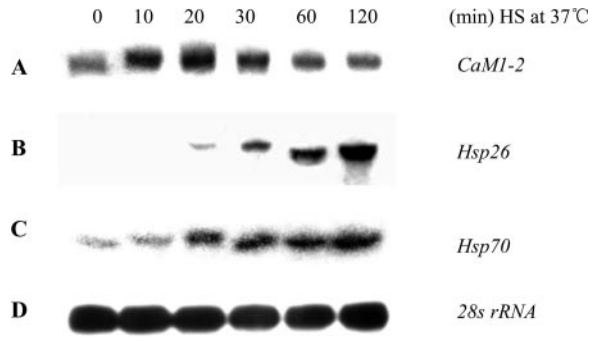

Figure 6. The expression kinetics of *CaM1-2*, *hsp26*, and *hsp70* genes during HS at 37°C. The growth conditions were the same as described for Figure 2. The tissues were incubated in distilled water at 37°C for the times indicated above each lane. Total RNA was extracted and analyzed by northern blot. Each filter was hybridized with the probe indicated on the right.

and peaked after 15 min of HS (Fig. 7). These results suggest that 5 min of HS is adequate to switch a key factor in the cell and initiate a signal transduction process that can carry on at a normal temperature (22°C for wheat).

DISCUSSION

Increase of $[Ca^{2+}]_i$ and CaM Gene Expression Induced by HS

In plant cells, the list of messengers used by signaling pathways includes Ca^{2+}, lipids, pH, and cyclic GMP (Sanders et al., 1999). However, no single messenger has been demonstrated to respond to more stimuli than cytoplasmic free Ca^{2+}. Environmental stimuli such as water stress, cold, wind, mechanical stimuli, and wounding cause increase of $[Ca^{2+}]_i$ (Knight et al., 1991, 1996, 1997). CaM appears to be ubiquitous among eukaryotes and is thought to be involved in fundamental cellular processes because of its extraordinary sequence conservation (Roberts and Harmon, 1992). As a mediator protein of Ca^{2+} signal, CaM is activated by binding Ca^{2+}, inducing a cascade of regulatory events. The expression of CaM or CaM-related genes is induced by many environmental stresses such as wind, touch, cold shock, pathogen attack, and wounding in plants (Braam, 1992; Depege et al., 1997; Heo et al., 1999; van der Luit et al., 1999).

A significant change in $[Ca^{2+}]_i$ induced by HS has been reported in both animal (Calderwood et al., 1988) and plant cells (Biyaseheva et al., 1993; Gong et al., 1998). Our results are consistent with their findings; however, in addition, we examined the kinetics of the changes in $[Ca^{2+}]_i$ during HS at 37°C in wheat cells. An important finding reported herein is that the increase in $[Ca^{2+}]_i$ took place only 1 min after HS and peaked after 4 min (Fig. 1, D and E). The accumulation of the CaM protein in the wheat seedlings was also induced by Ca^{2+} (Fig. 2), confirming a previous

report in maize seedlings (Gong et al., 1997b). In addition, expression of the wheat *CaM1-2* gene was up-regulated only 10 min after HS (Fig. 6A), later than the increase in $[Ca^{2+}]_i$ but earlier than HSP gene expression. Further results showed that 10 to 50 mM $CaCl_2$ increased the expression level of the wheat *CaM1-2* gene at non-HS temperature (22°C), indicating the dependence of CaM gene expression on $[Ca^{2+}]_i$ (Fig. 3). Possible roles of $[Ca^{2+}]_i$ in CaM gene expression have been documented (Polisensky and Braam, 1996; Depege et al., 1997; Heo et al., 1999). The rapid change in $[Ca^{2+}]_i$ implies that this increase could be a very early step in HS signal transduction. Expression of the CaM gene at an early stage of HS response and the dependence of CaM gene expression on Ca^{2+} confirm that the involvement of the Ca^{2+}-CaM signal system is upstream of HS signal transduction.

Involvement of Ca^{2+}-CaM in the Expression of HSPs during HS

The HS response is ubiquitous when cells are exposed to elevated temperatures. However, little is known about how the HS signal is perceived and transduced to activate the genes encoding the HSPs. Ca^{2+} and CaM are proposed to be important components upstream in HS signal transduction due to the rapid response of Ca^{2+} and CaM to HS. More studies are needed to verify this proposal.

The involvement of Ca^{2+} in activation of HSF (Mosser et al., 1990) and HSP synthesis (Kiang et al., 1994; Kuznetsov et al., 1998) has been reported, but the involvement of Ca^{2+} and CaM in expression of HSP genes or synthesis of HSPs has not been reported previously. The northern analysis results showed that $CaCl_2$ increased the mRNA level of the *hsp70* and *hsp26* genes and the calcium ion chelator EGTA, and the calcium ion channel blockers, $LaCl_3$ and verapamil, lowered them (Fig. 4, B–D). The expression of *hsp26* and *hsp70* genes was decreased by treatment with the CaM antagonist W7 or CPZ, but W5, an inactive structural analog of W7, did not affect the expression of HSP genes (Fig. 4, E–G). Treatment with $CaCl_2$ increased the synthesis of wheat HSPs remarkably, and removal of Ca^{2+} by EGTA lowered the synthesis of HSPs. The synthesis of HSPs was also lowered by treatment with the CaM antagonists CPZ or TFP in wheat (Fig. 5). In addition, we found that 10 to 50 mM $CaCl_2$ not only increased the expression level of the wheat *CaM1-2* gene but also up-regulated the expression level of the wheat *hsp26* gene under non-HS conditions. This means that increasing $[Ca^{2+}]_i$ instead of HS is able to induce the expression of HSP genes (Fig. 3B).

Some compounds such as EGTA, La^{3+}, and all other inhibitors were used to investigate the role of Ca^{2+} and CaM in this study. These compounds do affect living cells. In particular, La^{3+} has been shown to drastically perturb $[Ca^{2+}]_i$ homeostasis (Plieth 2001). Control experiments were performed before these compounds were used (for example, see Fig. 4, A and H). The results of control experiments showed these compounds in the concentration range we used under non-HS condition did not affect expression of genes used in this research. Employment of inhibitors such as verapamil and W7 should be feasible to study the role of Ca^{2+} and CaM in the cells. Considering their low specificity, further research such as the proofs in vivo is going on.

Evidence is given that there is considerable interlinking between heat and oxidative stress responses (Gong et al., 1997; Dat et al., 1998; Larkindale and Knight, 2002; Panchuk et al., 2002). However, the oxidative stress induced by HS is very weak under our experimental condition. First, we found in our previous experiments that mild heat treatment increased superoxide dismutase and catalase activities and also enhanced membrane thermostability in the wheat tolerant cultivar, 90-80, but not in the wheat sensitive cultivar (Zhou et al., 1995). The membrane injury caused by oxidative stress during heat treatment is lower in the tolerant cultivar than in the sensitive cultivar (Zhou et al., 1995). In this experiment, the tolerant cultivar 90-80 was used as our material. Second, our experimental results indicated that the malondialdehyde in wheat seedlings showed no significant increase at 37°C treatment for 0 to 120 min (data not shown). The change of $[Ca^{2+}]$ was measured within 10 min of HS, and the increase of *CaM1-2*, *hsp26*, and *hsp70* gene expression appeared within 20 min after HS in this study. The oxidative stress was negligible under this condition, and the changes of $[Ca^{2+}]$ and gene expression patterns shown in this study are mainly due to heat stress.

The Temporal Expression of the CaM1-2, hsp26, and hsp70 Genes

Our experimental results establish the kinetics of the $[Ca^{2+}]_i$ increase induced by HS and the expression of wheat *CaM1-2*, *hsp26*, and *hsp70* genes. These results define the order of the signal transduction steps during and immediately after HS. The increase of $[Ca^{2+}]_i$ induced by HS at 37°C occurs very quickly, taking only 1 min after HS (Fig. 1). The level of *CaM1-2* mRNA significantly increased 10 min after HS, but the increase in expression of *hsp26* and *hsp70* was detected 20 min after HS (Fig. 6). The expression of *CaM1-2* appears to increase more rapidly than expression of the HSP genes. The different temporal expression between CaM and HSP genes indicates that CaM is located upstream in HS signal transduction.

Only 5 min of HS at 37°C was needed to induce *hsp26* gene expression if a recovery time of 55 min at 22°C after HS was allowed (Fig. 7). However, a longer time of 20 min HS at 37°C was needed for

expression of *hsp26* gene if without recovery time (Fig. 6B). This result is consistent with previous work done with soybeans (*Glycine max*) by Kimpel et al. (1990) in which 7 min HS at 40°C followed by 113 min of recovery at 28°C caused expression of soybean HSP genes. These results indicate that 5 min of HS at 37°C is sufficient to activate a key factor in the cell and turn on the HS signal transduction pathway. We consider that $[Ca^{2+}]_i$ is probably the key factor because the increase of $[Ca^{2+}]_i$ induced by HS takes place within 1 min after HS, and $[Ca^{2+}]_i$ reaches its steady-state level 4 min after HS.

Our previous work has shown that there is a CaM-binding site within maize cytoplasmic HSP70 and that HSP70 binds CaM in a Ca^{2+}-dependent manner (Sun et al., 2000). The conservation of the CaM-binding sequence in cytoplasmic HSP70 family members from eukaryotes implies that the binding of CaM to HSP70 could have an essential biological function. CaM is a regulatory protein involved in a variety of cellular calcium-dependent signaling pathways. The functions of CaM are performed mainly through binding to target proteins (Roberts and Harmon, 1992). HSP70 is a potential autoregulatory factor (Shi et al., 1998; Bonner et al., 2000); therefore, we consider that CaM might play a regulatory function during the expression of HSPs through the binding directly to cytoplasmic HSP70. An increase in the Ca^{2+} and CaM concentration induced by HS enhances the binding of CaM to cytoplasmic HSP70, causing the HSF-HSP70 complex to release HSF, which binds to the HS element and activates transcription of HSP genes.

It is possible that there are several different pathways of HS signal transduction in cells. Mosser et al. (1990) suggested that HSF is activated directly by a conformational change caused by calcium or other biochemical conditions. The activation of HSF induced by denatured proteins under HS has been documented. Based on our findings herein, we propose a Ca^{2+}-CaM pathway of HS signal transduction. The HS signals are perceived by an as yet unidentified receptor. Receptor activation is closely followed by an increase in $[Ca^{2+}]_i$ through opening of Ca^{2+} channels in the plasma membrane or intracellular Ca^{2+} store membrane. This elevated level of cytoplasmic $[Ca^{2+}]_i$ then directly activates CaM and promotes the expression and accumulation of CaM. Activated CaM promotes the DNA-binding activity of HSF. Activation of HSF initiates the transcription and translation of HSP genes. Here, we propose two possible mechanisms by which active CaM might regulate the DNA-binding activity of HSF. One is through the binding of CaM directly to cytoplasmic HSP70. The other pathway is the regulation of HSF phosphorylation by regulation of CaM-dependent kinase activity. The mechanism of how CaM regulates activation of HSF to initiate the expression of HSPs remains to be solved and is the subject of ongoing studies in our laboratory.

MATERIALS AND METHODS

Reagents

All enzymes were purchased from Promega (Madison, WI) or Sino-American Biotechnology Company (Luoyang, People's Republic of China). Both the reverse transcription-PCR system and agarose are from Promega. W7, W5, CPZ, TFP, and verapamil were obtained from Sigma (St. Louis). Nylon membranes were the product of Gelman Instrument Co. (Ann Arbor, MI). [^{35}S]-Met was the product of Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). [α-^{32}P]dCTP was from the Beijing Yahui Bio-medical Technology Company (Beijing, People's Republic of China). The Random Primer DNA Labeling Kit was from the Beijing Dingguo Biotechnology Development Center (Beijing, People's Republic of China). All other reagents used were of analytical purity.

Plant Growth and Treatments

Seeds of winter wheat (*Triticum aestivum* L. cv 90-80) were imbibed overnight (12 h) in distilled water at 22°C. The soaked seeds were sown on three layers of filter paper wetted with distilled water for germination in the dark at 22°C for 3 d. Segments (1.5 cm long) excised from 3-d-old etiolated seedlings were used in all experiments. The tissue segments were placed wound-side down in 1 mL of different solutions (distilled water as control, $CaCl_2$, EGTA, verapamil, $LaCl_3$, W7, W5, CPZ, and TFP, respectively; the concentrations of compounds are described in the figure legends) at 22°C for 20 min, and then the tissue was subjected to a direct HS by placing in a controlled temperature incubator at 37°C for 1 h. In the experiments on kinetics of gene expression during HS, tissue incubated in distilled water was heat shocked in an incubator at 37°C for different times. In the recovery experiment, the tissue was heat shocked at 37°C for different times, then returned to 22°C. All treated tissues were immediately frozen in liquid N_2.

Measurement of $[Ca^{2+}]_i$

Tissue sections of 1.5 cm from 3-d-old etiolated wheat seedlings were used in all experiments except measurement of $[Ca^{2+}]_i$. For measurement of $[Ca^{2+}]_i$, a thin tissue section was needed to load the dye and make the LSCM observations. A thin segment with intact cell layers could be obtained from the leaf sheath of 10-d-old green wheat seedlings. We had to use the 10-d-old green seedlings for investigation of $[Ca^{2+}]_i$, although the 3-d-old wheat etiolated seedlings were used in all other experiments. The seedlings were grown in pots in growth chamber at 22°C day/18°C night under a fluorescent light (approximately 250 μmol m^{-1} s^{-1}) with a 12-h photoperiod. Fluo-3/AM was used as the Ca^{2+}-sensitive fluorescent probe. Thin tissue sections, about three cell layers thick and 0.5 cm long, were stripped from the sheath of the first leaf of a 10-d-old green seedling and rinsed with an isotonic solution three times, then incubated in medium containing 10 μM Fluo-3/AM at 24°C in the dark for 2 h. A section loaded with Fluo-3/AM was placed on outer surface of a glass tube, through which water was circulated from a bath with the aid of a pump. The temperature of the tissue on the outer surface of the glass tube was able to reach 37°C within 2 min and maintained at 37°C, whereas the warm water was circulated from water bath at 39°C into an inner glass tube. The subepidermal leaf sheath cells were observed under LSCM (MRC-1024 with a four-line argon laser box, Bio-Rad Laboratories, Hercules, CA). Excitation filter (488 ± 10 nm) and emission filter (530 ± 40 nm) were used in this experiment. The scan mode was XY-T (three dimensional). The change of fluorescence intensity in the cells with time was recorded with the Lasersharp 2000 time lapse program (Bio-Rad Laboratories). After that, the kinetics of fluorescence intensity were measured with the software Laserpix 4.0 (Bio-Rad Laboratories).

Preparation of Probes for Northern Analysis

The plasmid encoding the soybean (*Glycine max*) *hsp70* cDNA was kindly provided by Professor Joe L. Key (Botany Department, University of Georgia, Athens; Roberts and Key, 1991). The plasmid encoding the wheat

CaM1-2 cDNA was a gift from Professor Hillel Fromm (Weizmann Institute of Science, Rehovot, Israel; Yang et al., 1996). The plasmid encoding the soybean *hsp70* cDNA was digested with *Pst*I. The 5′ end of *hsp70* cDNA sequence, an approximately 2,000-bp DNA fragment, was isolated, purified, and used as an *hsp70* probe. The *CaM1-2* cDNA was obtained by PCR with primer T3 (ATTAACCCTCACTAAAGGGA) and T7 (TAATACGACTCACTATAGGG) and used as probe in northern analysis.

According to the published sequence (Joshi et al., 1997), the 3′ non-coding region of wheat *hsp26* cDNA was obtained by reverse transcription-PCR reaction with forward primer (CGGAATTCCGTATGTGCGA GACTG) and reverse primer (CGGAATTCCGATGCAGTAATTAA). The PCR product was then purified, digested with *Eco*RI, and ligated with pUC19 previously digested with *Eco*RI. The *hsp26* probe was obtained by the PCR reaction. The plasmid encoding the maize (*Zea mays*) rRNA gene was digested with *Eco*RI. An approximately 600-bp DNA fragment was isolated, purified, and used as the control probe.

Northern Analysis

The tissues frozen in liquid N_2 were ground with a mortar and pestle. The extraction of total RNA was performed essentially as described by Ausubel et al. (1998). Electrophoresis of RNA through formaldehyde-containing 1.2% (w/v) agarose gels, transfer onto Hybond N^+ nylon membranes, and hybridization with DNA probe were according to established methods (Sambrook et al., 1989). The DNA probes were labeled with [α-^{32}P]dCTP using a Random Prime DNA Labeling Kit. Prehybridization and hybridization were accomplished at 42°C for 4 and 16 h. respectively. The hybridized membranes were subjected to autoradiography after washing.

Determination of CaM Protein Level in Wheat Seedlings

For isolation of CaM, wheat tissue treated with Ca^{2+} or EGTA at 37°C was ground in liquid N_2, then homogenized in buffer (50 mM Tris-HCl [pH 8.0], 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM $NaHSO_4$, and 0.15 M NaCl) at 1:1 (w/v). The homogenates were disintegrated by sonication for total of 2 min, treated in a water bath of 90°C to 95°C for 3 min followed by cooling, then centrifuged at 10,000*g* for 30 min. The supernatants were used for measurement of protein quantity and CaM concentration. It has been reported that the content of apoplastic CaM is only 2.7% of total CaM in cells (Ye and Sun, 1988); therefore, total CaM was assumed to be cytoplasmic CaM in this experiment. Protein was quantified by the Bradford method using bovine serum albumin as the standard. The CaM concentration was determined by ELISA according to Sun et al. (1995). Recombinant potato (*Solanum tuberosum*) CaM was purified to electrophoretic homogeneity from *Escherichia coli* by a phenyl-Sepharose affinity chromatography. The antiserum against potato CaM was described by Bai et al. (2002).

In Vivo Labeling and Gel Electrophoresis

Wheat tissues were incubated in 1 mL of various treatment solutions (as described in the figure legends) and heat shocked at 37°C or kept at 22°C as control with gentle shaking. Forty microcuries of [^{35}S]-Met was added to each sample after 2 h at 37°C. Labeling was carried out for another 2 h at 37°C. The labeled tissues were rinsed thoroughly with rinse buffer (1 mM K-PO_4 [pH 7.5], 1% [w/v] Suc, and 5 mM Met), then immediately placed into liquid N_2. For protein isolation, the tissues were ground in liquid N_2 and then homogenized in homogenization buffer (50 mM Tris-HCl [pH 7.5], 2% [w/v] β-mercaptoethanol, and 5 mM EDTA). Homogenates were centrifuged at 12,000*g* at 4°C for 20 min. The supernatants were separated by 12.5% (w/v) SDS-PAGE. After gels were dried under vacuum, autoradiography was performed using Kodak x-ray film at −80°C.

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