Molecular and Genetic Evidence for the Key Role of AtCaM3 in Heat-Shock Signal Transduction in Arabidopsis

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Heat shock (HS) is a common form of stress suffered by plants. It has been proposed that calmodulin (CaM) is involved in HS signal transduction, but direct evidence has been lacking. To investigate the potential regulatory function of CaM in the HS signal transduction pathway, T-DNA knockout mutants for AtCaM2, AtCaM3, and AtCaM4 were obtained and their thermotolerance tested. Of the three knockout mutant plants, there were no differences compared with wild-type plants under normal conditions. However, the AtCaM3 knockout mutant showed a clear reduction in thermotolerance after heat treatment at 45°C for 50 min. Overexpression of AtCaM3 in either the AtCaM3 knockout or wild-type background significantly rescued or increased the thermotolerance, respectively. Results from electrophoretic mobility-shift assays, real-time quantitative reverse transcription-polymerase chain reaction, and western-blot analyses revealed that, after HS, the DNA-binding activity of HS transcription factors, mRNA transcription of HS protein genes, and accumulation of HS protein were down-regulated in the AtCaM3 knockout mutant and up-regulated in the AtCaM3-overexpressing transgenic lines. Taken together, these results suggest that endogenous AtCaM3 is a key component in the Ca2+-CaM HS signal transduction pathway.

In most organisms, temperatures above those needed for normal growth act as a form of stress. In plants, heat shock (HS) affects growth and crop production. Most organisms, including higher plants, synthesize heat shock proteins (HSPs) in response to high-temperature stress (Vierling, 1991; Queitsch et al., 2000; Nieto-Sotelo et al., 2002; Charng et al., 2006; Yang et al., 2006). In eukaryotes, expression of HSPs is regulated by heat shock transcription factors (HSFs). Upon HS, HSFs become activated and initiate the transcription of HSPs through binding to heat shock promoter elements (HSEs) in the promoter regions of genes encoding HSPs (Nover et al., 2001; Mishra et al., 2002; Baniwal et al., 2004; Charng et al., 2007). Many HSFs and HSPs have been shown to be involved in improving plant thermotolerance (Queitsch et al., 2000; Mishra et al., 2002; Wunderlich et al., 2003; Sanmiya et al., 2004; Charng et al., 2006, 2007; Nishizawa et al., 2006). Although the downstream components (HSFs, HSEs, and HSPs) of heat stress response have been studied in detail, the signal transduction pathway through which HS influences the binding activity of HSFs to HSEs to regulate HSP expression is not well understood. The aim of this study was to identify possible upstream components of HS signal transduction.

Several hypotheses regarding the HS signal transduction pathway have been proposed. For example, Ananthan et al. (1986) suggested that the accumulation of proteins denatured by heat stress stimulates the expression of HSP genes. Alternatively, changes in membrane fluidity and calcium influx following heat stress have also been reported (Sangwan et al., 2002; Sung et al., 2003). More recently, other authors have described potential roles for the different signaling molecules (Larkindale and Huang, 2004; Vaccar et al., 2004, 2006; Larkindale et al., 2005; Liu et al., 2006; Kotak et al., 2007).

In both animals and plants, data indicate that HS can elicit changes in the levels of intracellular Ca2+, one of the most ubiquitous cellular second messengers. Gong et al. (1998b) directly observed that HS caused a significant, transient increase in the intracellular concentration of free calcium ([Ca2+]i) in tobacco (Nicotiana plumbaginifolia). Increased [Ca2+]i was suggested to regulate the binding activity of HSFs to HSEs (Mosser et al., 1990; Li et al., 2004) and to protect against HS-induced oxidative damage in Arabidopsis (Arabidopsis thaliana; Larkindale and Knight, 2002). Our laboratory has reported evidence in wheat (Triticum aestivum) for...
an increase in \([\text{Ca}^{2+}]_i\) as early as 1 min after HS, with a maximal \([\text{Ca}^{2+}]_i\) at 4 or 10 min after HS in wheat or suspension-cultured Arabidopsis cells, respectively (Liu et al., 2003, 2006).

Calmodulin (CaM) is a well-studied intracellular calcium sensor that mediates \(\text{Ca}^{2+}\) signal transduction (Snedden and Fromm, 1998, 2001). Gong et al. (1997b) observed that CaM was up-regulated during HS in maize \((\text{Zea mays})\) seedlings. In our studies, the levels of \(\text{CaM}\) mRNA and protein increased during HS in wheat, and the increase of \(\text{CaM}\) mRNA for only 10 min preceded the expression of HSP genes, which was detected after 20 min of HS (Liu et al., 2003). We also reported that CaM antagonists, including CPZ, W7, and CaM antiserum, inhibited the DNA-binding activity of maize HSF during HS (Li et al., 2004). More recently, a CaM-binding protein kinase was identified as an important component in the \(\text{Ca}^{2+}\)-CaM pathway involved in HS signal transduction (Liu et al., 2008). Collectively, available evidence suggests that AtCaM may participate as a key component in HS signal transduction. However, to date, there is no direct genetic evidence for CaM involvement in HS signaling.

Arabidopsis contains at least nine different CaM genes. \(\text{AtCaM1}\) through \(\text{AtCaM7}\) share a high level of sequence identity (>95%), differing from each other only by a few nucleic acids; \(\text{AtCaM8}\) and \(\text{AtCaM9}\) are more divergent from the other forms (Reddy et al., 2000; Luan et al., 2002). Our previous studies started with all nine members of the \(\text{AtCaM}\) gene family, but only \(\text{AtCaM3}\) gene expression showed a significant up-regulation after a 37°C HS. Moreover, the temporal expression of the \(\text{AtCaM3}\) and \(\text{AtHsp18.2}\) genes demonstrated that up-regulation of \(\text{AtCaM3}\) expression occurred earlier than that of \(\text{AtHsp18.2}\) (Liu et al., 2005). These results suggested \(\text{AtCaM3}\) may be involved in the HS signal transduction pathway. Also, based on the gene expression results (supplemental data) and the existing mutants, \(\text{AtCaM2}\) and \(\text{AtCaM4}\) were selected as the controls.

In this paper, we generated T-DNA knockout mutants and transgenic plants to provide direct molecular genetic evidence that endogenous \(\text{AtCaM3}\) is a key component in HS signal transduction. We also confirmed the existence of a previously proposed, new \(\text{Ca}^{2+}\)-CaM HS signal transduction pathway (Liu et al., 2003, 2008).

RESULTS

\(\text{AtCaM3}\) T-DNA Insertion Mutant Decreases the Thermotolerance of Arabidopsis Seedlings

To determine whether \(\text{AtCaM3}\) is involved in the HS signal transduction pathway, we first utilized a reverse genetic approach by screening Arabidopsis T-DNA insertion mutants effecting \(\text{AtCaM}\) family members. T-DNA insertion mutants for \(\text{AtCaM}\) genes were obtained from the Arabidopsis Biological Resource Center. Of all the mutants obtained, only the homozygous T-DNA insertion mutants \(\text{cam2, cam3,}\) and \(\text{cam4}\), which separately affect \(\text{AtCaM2, AtCaM3,}\) and \(\text{AtCaM4}\), respectively, were detected to be deficient in transcripts by reverse transcription (RT)-PCR analysis in this experiment (Fig. 1B). The positions of the individual T-DNA insertions are shown in Figure 1A.

\(\text{AtCaM2}\) and \(\text{AtCaM4}\) were used as controls and compared with \(\text{AtCaM3}\) in a thermotolerance assay. The \(\text{cam2, cam3,}\) and \(\text{cam4}\) mutants exhibited no phenotypic differences compared with wild-type plants under normal growth conditions (Fig. 2A). However, the thermotolerance assay in which 6-d-old seedlings were heat shocked at 45°C for 50 min and then allowed to recover for 6 d at 22°C showed that thermotolerance of the \(\text{cam2}\) and \(\text{cam4}\) mutant seedlings was similar to that of the wild-type seedlings and that only the \(\text{cam3}\) mutant exhibited a significant impairment in thermotolerance (Fig. 2A). The survival rate of the \(\text{cam3}\) mutants was about 43% lower than that of the wild-type plants (Fig. 2B).

With the exception of gene expression analyses by RT-PCR (Fig. 1B), to further determine whether the reduction in \(\text{AtCaM3}\) was responsible for the increased heat sensitivity, we examined the endogenous total \(\text{AtCaM}\) protein (translated by all \(\text{AtCaM}\) genes) levels in wild-type plants and in \(\text{cam2, cam3,}\) and \(\text{cam4}\) mutants after HS at 37°C by western blot. As shown in Figure 2C, \(\text{AtCaM}\) protein was lower in the \(\text{cam3}\) mutants.
mutant than in wild-type plants or the cam2 and cam4 mutants.

**Effects of HS on the Expression of AtCaM3 and the Expression Pattern of AtCaM3**

The expression of AtCaM3 after HS was studied in Arabidopsis in order to gain insight into the function of its protein product. The AtCaM3 promoter was fused to the reporter gene GUS and introduced into Arabidopsis by Agrobacterium tumefaciens-mediated transformation. AtCaM3::GUS transgenic plants were used to analyze the expression pattern of AtCaM3 under HS conditions. Four different transgenic lines were tested. Plants were grown at 22°C for 10 d and then treated at 37°C before they were harvested for GUS activity assays. Assay results showed that, compared with the activity at 0 h, HS treatment caused GUS activity to increase rapidly by 2-fold in the first 2 h. When heat treatment was extended to 4 h, GUS activity was slightly lower than was observed for the 2-h treatment but was still 1.8-fold higher than at 0 h (Fig. 3A). These GUS activity assays confirmed the results of previous real-time RT-PCR experiments, in which the expression of the AtCaM3 gene was significantly up-regulated after HS (Liu et al., 2005). Based on these results, 2 h of HS was used as a reference for increasing AtCaM3 in further experiments (Fig. 2C).

We also examined the tissue- and organ-specific expression of AtCaM3 in Arabidopsis. As shown in Figure 3B, AtCaM3::GUS was expressed ubiquitously in young seedlings as well as in rosette and cauline leaves (Fig. 3B, a–c). When plants entered the flowering stage, AtCaM3::GUS expression became concentrated in the sepals, stigma, and filaments but not in the anthers or petals (Fig. 3B, e–h). AtCaM3::GUS was also strongly expressed in the separation layer of the abscission zone of flower organs in which the petal, sepal, and stamen had fallen off after anthesis (Fig. 3B, d and i). These results are consistent with the results of previous microarray analyses (Zimmermann et al., 2004). GUS assays revealed that AtCaM3 was expressed in most organs and tissues of the plant, especially in the young seedling.

**The Expression of AtCaM3 in the cam3 Mutant Rescues Heat Sensitivity**

To confirm whether the suppression of AtCaM3 gene expression in cam3 was responsible for the phenotype of impaired thermotolerance, we also performed complementation analyses. A binary vector containing the coding region of AtCaM3 under the control of the 35S promoter was constructed and used to transform the cam3 mutant by the floral-dip method. Due to the more than 95% nucleic acid sequence identity of the seven
members, to ensure the specificity of the primers, the NOS terminator region of the binary vector was used to design the reverse primer used to identify the transgenic plants. RT-PCR showed that ectogenous \textit{AtCaM3} expression was only found in the complemented transgenic lines. By contrast, ectogenous \textit{AtCaM3} cDNA was not amplified in the wild-type or \textit{cam3} plants (Fig. 4A). These data confirmed that the \textit{cam3} mutant was successfully transformed with the \textit{AtCaM3} cDNA. Six homozygous lines of complemented transgenic seedlings (\textit{cam3}/\textit{AtCaM3}) were obtained and identified. Three of these lines (1-2, 7-4, and 43-1) were chosen for thermotolerance studies. In these experiments, none of the \textit{cam3}/\textit{AtCaM3} transgenic plants showed variant phenotypes under normal growth conditions as compared with the wild-type and \textit{cam3} plants (Fig. 4B). However, the \textit{cam3}/\textit{AtCaM3} transgenic lines completely rescued the heat-hypersensitive phenotype of the \textit{cam3} mutants (Fig. 4C) and exhibited survival rates that were similar to those of the wild-type plants (Fig. 4D).

It has been reported that heat induces oxidative damage, and considerable interlinking between heat and oxidative stress responses have been suggested (Gong et al., 1997a, 1998a; Dat et al., 1998). As an additional test of heat sensitivity, the \textit{cam3} mutants and complemented transgenic lines were assayed for the accumulation of thiobarbituric acid-reactive substances (TBARS) following heat stress. Plant tissues used in TBARS assays were harvested 2 d after HS at 45°C for 1 h (Larkindale and Knight, 2002; Larkindale et al., 2005). Damage to seedlings, including bleached leaves and plant bleaching, suggested that the damage caused by heating may have been due to oxidative stress occurring during the recovery phase. TBARS levels in three \textit{cam3}/\textit{AtCaM3} transgenic lines were similar to those detected in wild-type plants (Fig. 4E). However, TBARS levels in \textit{cam3} mutant plants, which showed decreased thermotolerance, were 250% higher than those detected in wild-type plants (Fig. 4E).

\textbf{Overexpression of AtCaM3 Improves the Thermotolerance of Arabidopsis Seedlings}

To further confirm the function of \textit{AtCaM3} in thermotolerance, transgenic plants were generated by transformation of the \textit{AtCaM3} gene fused to GUS under the control of the 35S promoter (35S::\textit{AtCaM3}-\textit{GUS}) in the ecotype Columbia background. Because of the high level of nucleic acid sequence identity among \textit{AtCaM} multigene family members, no specific primers could be used to determine the expression level of \textit{AtCaM3} by real-time RT-PCR. Thus, GUS was used as a label to show \textit{AtCaM3} expression levels in the different transgenic lines. After testing the level of \textit{AtCaM3} expression (as indicated by GUS activity) of seven independent T3 homozygous transgenic lines, three lines (3-7, 3-21, and 3-22) with strong GUS activity compared with wild-type control plants (Fig. 5A) were used in the following experiments. Transgenic plants carrying 35S::GUS were used as a vector control.

The three 35S::\textit{AtCaM3}-\textit{GUS} homozygous transgenic lines, a vector control line, and wild-type plants were plated together on the same Murashige and Skoog plate. There were no significant phenotypic differences between the transgenic and wild-type plants under normal growth conditions (Fig. 5B). The seedlings were grown at 22°C for 6 d followed by exposure to 45°C for 70 min, then they were allowed to
recover at 22°C for another 6 d. As shown in Figure 5C, most of the wild-type and vector control plants could not survive under this lethal-level heat stress. The cotyledons of these plants were bleached and dried. However, although they all exhibited a delay in growth, seedlings overexpressing AtCaM3 survived, as demonstrated by their green cotyledons and the presence of young leaves (Fig. 5C). The overall survival rate of the AtCaM3-GUS-overexpressing transgenic lines was 40% to 60% higher than in either the wild-type or vector control plants (Fig. 5D). In the three AtCaM3-GUS-overexpressing transgenic lines, TBARS levels were 24% to 40% lower than those in either the wild-type plants or the 35S::GUS vector control plants (Fig. 5E).

Effect of AtCaM3 on DNA-Binding Activity of HSF and Expression of HSP Genes during HS

In order to understand the underlying mechanism of AtCaM3-induced thermotolerance in Arabidopsis, the binding activity of HSF to HSE in AtCaM3 knockout mutant, AtCaM3-GUS-overexpressing, and wild-type plants was analyzed by electrophoretic mobility-shift assay. Results indicate that after HS the binding activity of HSF to HSE in the cam3 mutant plants was weaker than that detected in the wild-type plants. In the three AtCaM3-GUS-overexpressing transgenic lines (3-7, 3-21, and 3-22), the binding activity was much stronger than in the wild-type plants (Fig. 6A). As a control, there was no binding when the wild-type plant was not heated, which suggested that the band shift was induced specifically by heat (Fig. 6A). These data indicate that changes in AtCaM3 expression influence the binding activity of HSF to HSE.

The effect of AtCaM3 on the transcriptional regulation of HSPs was also examined by real-time quantitative RT-PCR. AtHSP18.2, AtHSP25.3, and AtHSP83 were chosen as marker genes. After HS at 37°C for 1 h, levels of AtHsp18.2 mRNA were 66% lower in the cam3 mutants than in the wild-type plants, while levels in the three cam3/AtCaM3 transgenic lines were rescued such that they were 56% to 63% higher than in the wild-type plants (Fig. 6B). Thus, overexpression of AtCaM3 increased the expression of AtHsp18.2. The relative level of AtHsp18.2
expression in the AtCaM3-GUS-overexpressing transgenic lines was 70% to 130% higher than in the wild-type plants (Fig. 6B).

Similar results were obtained for the expression of AtHsp25.3 and AtHsp83 in the mutant and different transgenic lines. After HS at 37°C for 1 h, AtHsp25.3 and AtHsp83 mRNA levels in the cam3 mutants were reduced to 47% and 64% of levels detected in the wild-type plants (Fig. 6, C and D), while complemented transgenic lines showed fully restored AtHsp25.3 and AtHsp83 expression (Fig. 6, C and D). Similarly, the expression of AtHsp25.3 and AtHsp83 in the AtCaM3-GUS-overexpressing transgenic lines was 40% to 160% higher than in the wild-type plants (Fig. 6, C and D).

The effect of AtCaM3 on the accumulation of AtHSP18.2 was further documented through examination of protein changes by western blot. Ten-day-old seedlings were heat treated at 37°C for 2 h or maintained at 22°C and cellular protein was extracted. AtHSP18.2, a heat-induced protein, was not detected in plants incubated at the normal growth temperature of 22°C. HS at 37°C for 2 h rapidly induced the expression of AtHSP18.2 protein (Fig. 7A). Similar to changes seen in RNA transcriptional regulation, AtHSP18.2 accumulation in the cam3 mutants was lower than in the wild-type plants (Fig. 7A). In the three cam3/AtCaM3 transgenic lines (1-2, 7-4, and 43-1), AtHSP18.2 accumulation was rescued as compared with the cam3 mutant, with AtHSP18.2 levels that were similar to those in the wild-type plants (Fig. 7B). AtHSP18.2 levels in the three AtCaM3-GUS-overexpressing lines (3-7, 3-21, and 3-22) were higher than in the wild-type plants (Fig. 7C). In these experiments, tubulin protein expression was used to ensure equal sample loading. These results suggest a link between changes in the expression and accumulation of HSPs in both cam3 mutant and transgenic plants and changes in plant thermotolerance.

Figure 5. Overexpression of AtCaM3 improves thermotolerance. C, Vector control; WT, wild type; 3-7, 3-21, and 3-22 are three AtCaM3-GUS-overexpressing transgenic lines transformed with 35S:AtCaM3-GUS. A, GUS activity in wild type, vector control, and three transgenic lines. Each value is the mean ± se of three biological replicates. B and C, Wild-type, vector control, and three transgenic lines under normal (B) and HS (C) conditions. For the therмотolerance assay, 6-d-old seedlings grown at 22°C were shifted to 45°C for 70 min and then returned to 22°C for 6 d, at which time they were photographed. D, Survival rates for the wild-type, vector control, and three transgenic lines. Survival was determined 6 d after heat stress at 45°C for 70 min. Each value is the mean ± se of three biological replicates, 30 seedlings per experiment. E, TBARS levels of wild-type, vector control, and three transgenic lines. Plants were heat stressed at 45°C for 60 min and then allowed 2 d at 22°C for recovery. TBARS levels were normalized relative to the wild-type control. Each value is the mean ± se of three biological replicates.
DISCUSSION
The Effect of AtCaM3 on Thermotolerance in Arabidopsis

As one of the most conserved cellular proteins, CaM has been well studied in its role as an intracellular Ca2+ sensor. CaM participates in numerous signaling pathways, and expression of CaM genes in plants is regulated by many types of environmental stresses, including wind, touch, wounding, osmotic stress, pathogens, and cold stress (Braam et al., 1997; Jang et al., 1998; Bergey and Ryan, 1999; Heo et al., 1999; Reddy, 2001; Yamakawa et al., 2001; Townley and Knight, 2002; Yang and Poovaiah, 2003; McCormack et al., 2005). It has also been reported that HS could induce the accumulation of CaM (Gong et al., 1997b). In our previous studies, we provided preliminary, indirect evidence for the involvement of CaM in HS signal transduction (Liu et al., 2003, 2005; Li et al., 2004). In Arabidopsis, the AtCaM gene family contains nine members that share a high level of nucleic acid and amino acid sequence identity (Snedden and Fromm, 2001; Luan et al., 2002). Based on previous results regarding the expression of nine AtCaM genes and temporal expression of AtCaM3 and AtHsp18.2 (Liu et al., 2005), our attention was focused on AtCaM3. Here, compared with a no-heat control, we analyzed the expression time course of AtCaM2, AtCaM3, and AtCaM4 by real-time quantitative RT-PCR. The results indicated that the level of AtCaM3 mRNA increased and reached its maximum after 20 min of HS at 37°C, then decreased slowly after 30 min. The levels of AtCaM2 and AtCaM4 did not increase after HS treatment (Supplemental Fig. S4), so AtCaM2 and AtCaM4 were selected as the controls. The results in Supplemental Figure S4 and the data shown in Figure 3A together suggest that AtCaM3 is significantly induced by HS. Thus, AtCaM3 may be a component in HS signal transduction.

To obtain direct molecular genetic evidence for the function of AtCaM3 in HS signaling pathways, AtCaM3 T-DNA knockout mutants, complemented transgenic plants, and AtCaM3-GUS-overexpressing plants were generated. Thermotolerance testing indicated that knockout of AtCaM3 by T-DNA insertion clearly decreased the thermotolerance of Arabidopsis, whereas complementary transformants were able to completely rescue the decreased thermotolerance of the cam3 mutant (Fig. 4). Moreover, overexpression of AtCaM3 resulted in transgenic plants with a thermo-
tolerance much higher than that observed in wild-type plants (Fig. 5).

As shown in Figure 2, single AtCaM gene knockout (including the cam3 mutant) did not exhibit any phenotypic differences as compared with the wild-type plants under normal growth conditions. These data suggested the existence of functional redundancy between different AtCaM members for plant growth. But the absence of a single AtCaM3 gene or overexpression could change thermotolerance under HS conditions. As shown in Figure 3:GUS activity tests (Fig. 3A) and western-blots analyses (Fig. 2C), because AtCaM3 was heat induced, AtCaM3 protein in the AtCaM3 T-DNA insertion mutant was not up-regulated after HS. Using a polyclonal antibody, the cam3 mutant showed lower total AtCaM protein levels than the wild type and other mutant (cam2 and cam4) plants. These results suggested that higher AtCaM3 protein levels were linked to high thermotolerance.

Even though AtCaM2 and AtCaM4 have a high amino acid sequence identity with AtCaM3 (Supplementary Fig. S3; Supplemental Table S1), the cam2 and cam4 mutants did not show obvious differences in thermotolerance compared with wild-type plants after HS at 45°C, unlike the cam3 mutant (Fig. 2A). The reason for this phenotypic difference may be that different CaM genes are differentially regulated by distinct cis-regulatory elements under differing stress conditions. Moreover, even CaM genes encoding the same protein can be differentially regulated in response to different external stimuli, such as disease resistance and cold (Takezawa et al., 1995; Zielinski, 1998; Heo et al., 1999; Townley and Knight, 2002; Yang and Poovaiah, 2003). Because we did not obtain knockout mutants of all of the AtCaM genes, we cannot conclude that only AtCaM3 is involved in HS signal transduction. Nonetheless, our data provide strong evidence for AtCaM3 as one of the major components participating in the pathway involving Ca2+-CaM in HS signal transduction in Arabidopsis.

The Mechanism for the Effect of AtCaM3 on Thermotolerance

In eukaryotes, HSFs are the downstream components of the HS signal transduction chain, which regulates the expression of genes encoding HSPs. HSPs, in turn, are known to contribute to thermotolerance (Kotak et al., 2007). Nover et al. (2001) identified 21 open reading frames for HSFs in the Arabidopsis genome. It was demonstrated that HSF1a acts as a major regulator of the heat stress response in tomato (Solanum lycopersicum; Mishra et al., 2002). HSF phosphorylation has been proposed to play an important role in regulating the activity of this group of proteins (Schofl et al., 1998; Dai et al., 2000; Hashikawa and Sakurai, 2004; Wang et al., 2006). Wang et al. (2004) found that Arabidopsis CaM-binding protein kinase3 (AtCBK3) binds to CaM in a Ca2+-dependent manner and that this binding was directly regulated by the kinase activity of AtCBK3. Our recent work further showed that, as a CaM target protein, AtCBK3 activity could be stimulated by AtCaM (Supplemental Fig. S2). AtCBK3 phosphorylated AtHSA1a, thus regulating the binding activity of HSF to HSE (Liu et al., 2008). Here, we have provided evidence that the DNA-binding activity of HSFs was increased in Arabidopsis cells overexpressing AtCaM3 but decreased in the AtCaM3 knockout mutant (Fig. 6A). Therefore, AtCaM3 may modulate the thermotolerance of Arabidopsis by regulating the phosphorylation status affected by AtCBK3 and thus the activity of HSFs.

HSF binding to HSE activates the transcription of HSP genes (Baniwal et al., 2004; Hahn et al., 2004; Yamamoto et al., 2005), which are thought to encode molecular chaperones responsible for protein folding, assembly, translocation, and degradation (Miernyk, 1999; Hartl and Hayer-Hartl, 2002; Nollen and Morimoto, 2002; Mayer and Bukau, 2005). These proteins are classified based on their molecular masses and include HSP100, HSP90, HSP70, HSP60, and small HSPs. An
important class is the small HSPs. These small HSPs are probably critical for survival of heat stress and for specific developmental processes in plants (Waters et al., 1996). Hence, we selected two small HSPs and one HSP gene of the HSP90 family. Our previous work using CaM antagonist experiments suggested that CaM affected the expression of HSP genes (Liu et al., 2003). In this study, using transgenic plants, we examined the effect of AtCaM3 on the expression of two genes encoding small HSPs, HSP18.2 (Takahashi and Komeda, 1989) and HSP25.3 (Osteryoung et al., 1993), and one gene belonging to the HSP90 family, HSP83 (Takahashi et al., 1992). Our results indicate that down-regulation of AtCaM3 expression in mutant plants led to reduced expression of these HSP genes, while complementary transformants of the cam3 mutant showed restored expression of these genes to the levels observed in wild-type plants. Furthermore, overexpression of AtCaM3 increased HSP expression following HS (Fig. 6). HSP expression in the cam2 and cam4 mutants was not changed, which was consistent with the lack of change in thermotolerance experiments compared with wild-type plants (Supplemental Fig. S1). Furthermore, results of AtHSP18.2 protein accumulation analyses in Arabidopsis seedlings were similar to those obtained for the expression of the AtHSP18.2 (Figs. 6C and 7). Taken together, the mechanism by which AtCaM3 affects plant thermotolerance may involve regulation of the DNA-binding activity of HSFs and HSP gene expression and protein accumulation.

**AtCaM3 Is a Key Component in the Ca²⁺-CaM Pathway of HS Signal Transduction**

Several HS signal transduction pathways have been proposed. Ananthan et al. (1986) suggested that the accumulation of heat stress-denatured proteins stimulates the expression of HSP genes. In this model, the chaperone HSP70 is thought to form a protein complex with HSF under normal conditions. After HS, the denatured proteins produced by HS in cytoplasm bind to HSP70, releasing HSF from the complex and freeing it to activate HSP expression. The involvement of membrane fluidity and calcium-dependent signaling molecules in plant HS signal transduction has also been proposed. In this putative pathway, HS induces a change in membrane fluidity, Ca²⁺ influx, and cytoskeletal remodeling. The increased cytosolic calcium concentration was suggested to activate calcium-dependent protein kinase (or mitogen-activated protein kinase) cascades and subsequently initiate the HS signal transduction pathway (Sangwan et al., 2002; Sung et al., 2003). Recently, other authors have described potential roles for many different signaling molecules, including ethylene, abscisic acid, inositol 1,4,5-trisphosphate, salicylic acid, and hydrogen peroxide, in HS signaling pathways (Larkindale and Huang, 2004; Vacca et al., 2004; Larkindale et al., 2005; Liu et al., 2006; Volkov et al., 2006; Kotak et al., 2007).

We have proposed a Ca²⁺-CaM pathway involved in HS signal transduction (Liu et al., 2003). Our laboratory and others have reported that HS-induced [Ca²⁺]i influx, as a primary response, regulated the binding of HSF to HSE and the subsequent expression of HSPs (Mosser et al., 1990; Gong et al., 1998b; Li et al., 2004; Liu et al., 2006). More recently, we found that AtCBK3 is an important AtCaM downstream component of the HS signal transduction pathway. So AtCaM could be the key connection between upstream [Ca²⁺]i increases and downstream atCBK3 activation in this pathway, but direct genetic evidence for the participation of AtCaM3 is still missing. In this study, we used T-DNA knockout and AtCaM3-overexpressing plants to provide molecular and genetic evidence showing that AtCaM3 affects plant thermotolerance by regulating the DNA-binding activity of HSFs and the gene expression and protein accumulation of HSPs. Therefore, our data fill the gap between upstream changes in Ca²⁺ concentration and downstream activation of CaM-binding protein kinases, thus confirming the Ca²⁺-CaM pathway of HS signal transduction in Arabidopsis.

According to this model, the HS signal is perceived by an as yet unidentified receptor, leading to an increase in the cytosolic concentration of Ca²⁺, perhaps through the regulation of inositol 1,4,5-trisphosphate/ phospholipase C. This elevated [Ca²⁺]i directly activates AtCaM3 and, in turn, stimulates AtCBK3, which ultimately regulates the phosphorylation and DNA-binding activity of HSFs. By binding to HSEs, HSFs may initiate the transcription of HSP genes as part of the plant’s adaptation to environmental heat stress.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Seeds of Arabidopsis (Arabidopsis thaliana ecotype Columbia) were surface sterilized and plated on Murashige and Skoog medium containing 1.0% (w/v) Suc and 0.3% (w/v) Phytagel (Sigma). The seeds were vernalized at 4°C for 3 d and then grown under long-day conditions (16 h of light/8 h of dark) at 22°C.

The T-DNA insertion lines for AtCaM3 (SALK_001357) and AtCaM2 (SALK_114166) were obtained from the Arabidopsis Biological Resource Center. The T-DNA insertion line for AtCaM4 (GABI_309E99) was ordered from GABI-Kat. T-DNA insertion lines were screened and isolated as described by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdna_FAQs.html). Homozygous mutants were used in further analyses.

**Construction of Transgenic Lines**

The 35S cauliflower mosaic virus promoter (restriction enzymes PstI and XbaI) and the reporter gene GUS (restriction enzymes BamHI and SalI) were cloned into the vector pCAMBIA3000, yielding pCAMBIA1300M.

To generate AtCaM3:GUS transgenic plants, a fragment of the AtCaM3 promoter containing 1.2 kb of the upstream region of the gene was amplified by PCR using the forward primer 5'-AAGTCGACAGGGATTAAGGGC-CACAAC-3' and the reverse primer 5'-GCTCTAGACTTGTGCTAATAA-GCTGAAAG-3'. The amplified fragment was cloned into pCAMBIA1300M using the PstI and XbaI restriction sites, thus replacing the 35S promoter and generating the AtCaM3::GUS construct.

To generate 35S:AtCaM3 for complementation experiments, the AtCaM3 coding region was amplified by PCR from the AtCaM3 cDNA using the primers CaM3F1 (5'-CTCTAGAATGGCGGATCAGCTCACCGA-3') and
Calculated according to C = 6.45 (OD$_{532}$ - OD$_{600}$) – 0.56 OD$_{600}$, where OD$_{600}$ is the optical density at 532 nm. The TBARS levels in extracts from mutant or transgenic plants were determined relative to the level in wild-type extracts.

**Electrophoretic Mobility-Shift Assays**

The HSE (Scharf et al., 1990, 1998; Hüböl and Schöffl, 1994; Li et al., 2004) oligonucleotides (5'-TCAAGAGCTTCAGTCGAACTTCAGTAAAGCAGATT-3' and 5'-TGGAGATTCAGCCCTCAGGATGACGTGT-3') were annealed and labeled with [gamma-32P]ATP using T4 polynucleotide kinase (TaKaRa). Ten-day-old seedlings were ground in liquid nitrogen and extracted with buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 10 mM boric acid, and 0.1 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatants were used as whole cell extracts. These extracts were heat shocked at 37°C for 1 h. Electrophoretic mobility-shift assays were carried out according to the method of Li et al. (2004).

**Real-Time Quantitative RT-PCR Analysis**

Total RNA (500 ng) isolated using the Trizol reagent (Invitrogen) from 10-d-old seedlings was used together with the PrimeScript RT Reagent Kit (TaKaRa) for first-strand cDNA synthesis according to the manufacturer’s instructions. For RT-PCR, SYBR Premix Ex Taq (TaKaRa) was used. The PCR program was as follows: initial polymerase activation for 10 s at 95°C, and 40 cycles of 95°C for 5 s followed by 60°C for 30 s. The reactions were carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primer pairs were designed using Primer Express (Applied Biosystems). The primers used for AtHsp18.2 (At5g59720) were 5'-TCTGGTATGGCCTAGGCT-TA-3' (forward) and 5'-AAGTCCCGTTCGAAACATGTG-3' (reverse); those for AtHsp25.3 (At4g27670) were 5'-GACGTCTCTCTTCCGATTGTG-3' (forward) and 5'-CTCCACTCCCTCTCTTCTCT-3' (reverse); and those for AtHsp83 (At5g52640) were 5'-GCTCTTAGGATCCAGAGATT-3' (forward) and 5'-TCTCCATCTTGGTC TCTTCA-3' (reverse). The primers used for the internal control actin (At2g37620) were 5'-TGGTCTCATGGTG-GAACA-3' (forward) and 5'-GGGCGACGAGCAGATGCCTT-3' (reverse).

**Western Blot Analyses**

Ten-day-old seedlings were kept at 37°C for 2 h and then ground in liquid nitrogen. Total protein was extracted using extraction buffer (10 mM HEPES, pH 7.9, 0.4 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride), and the extracts were clarified by centrifugation at 14,000g for 20 min at 4°C. Supernatants were transferred to fresh tubes, and the protein content was determined (Bradford, 1976). Total proteins (40 μg) were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. Polyvinylidene difluoride membranes were blocked for at least 2 h and then probed with rabbit antisera against AtHsp18.2 and mouse antiserum against the loading control tubulin (Sigma). After extensive washing, membranes were incubated with the appropriate secondary antibodies conjugated to alkaline phosphatase. BCIP/NBT (Amresco) was used for immunodetection.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Effect of AtCaM3 on the expression of HSP genes and the accumulation of AtHsp18.2 in cam2 and cam4 mutants.

**Supplemental Figure S2.** The activation of additive CaM on the activity of A1CBK3.

**Supplemental Figure S3.** Alignment of the amino acid sequences of three AtCaM proteins; amino acid sequence identities are shown in red.

**Supplemental Figure S4.** The expression pattern of AtCaM2, AtCaM3, and AtCaM4 during HS at 37°C.

**Supplemental Table S1.** Protein data comparison for AtCaM2, AtCaM3, and AtCaM4.

**Supplemental Table S2.** Primer used for real-time quantitative RT-PCR.
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LITERATURE CITED


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