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The calmodulin-binding protein kinase 3 is part of heat-shock signal transduction in *Arabidopsis thaliana*

Hong-Tao Liu[1,2], Fei Gao[1,2], Guo-Liang Li[1], Jin-Long Han[1,2], De-LongLiu[3], Da-Ye Sun[2] and Ren-Gang Zhou[1,2,*]

[1]*Institute of Genetics and Physiology, Hebei Academy of Agricultural Sciences, Shijiazhuang 050051, China,*

[2]*Hebei Provincial Key Laboratory of Molecular Cell Biology, Hebei Normal University, Shijiazhuang 050016, China, and*

[3]*Academy of Chemistry, Hebei Normal University, Shijiazhuang 050016, China*

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*For correspondence (fax +86 311 87652335; e-mail rengangzhou@yahoo.com.cn).

Summary

Based on our previous findings, we proposed a pathway for the participation of Ca^{2+}/calmodulin (CaM) in heat-shock (HS) signal transduction. The specific mechanism by which CaM regulates activation of heat-shock transcription factors (HSFs) is not known. CaM-binding protein kinases (CBK) are the most poorly understood of the CaM target proteins in plants. In this study, using a yeast two-hybrid assay, we found that AtCBK3 interacts with AtHSFA1a. Fluorescence resonance energy transfer was used to confirm the interaction between AtCBK3–YFP and AtHSFA1a–CFP. Furthermore, we demonstrate that purified recombinant AtCBK3 phosphorylated recombinant AtHSFA1a *in vitro*. We also describe the results of both downregulation of *AtCBK3* expression and ectopic overexpression in *Arabidopsis thaliana*. The T-DNA insertion *AtCBK3* knockout lines had impaired basal thermotolerance, which could be complemented by transformation of plants with the native gene. Overexpression of *AtCBK3* resulted in plants with increased basal thermotolerance. Results from real-time quantitative PCR and protein gel-blot analyses suggest that AtCBK3 regulates transcription of heat-shock protein (HSP) genes and synthesis of HSPs. The binding activity of HSF to the heat-shock element (HSE), the mRNA level of HSP genes and synthesis of HSPs were upregulated in AtCBK3-overexpressing lines after HS, but downregulated in AtCBK3 null lines. These results indicate that AtCBK3 controls the binding activity of HSFs to HSEs by phosphorylation of AtHSFA1a, and is an important component of the HS signal transduction pathway.

Keywords: calmodulin-binding protein kinase, calmodulin, phosphorylation of HSF, thermotolerance, Arabidopsis, signal transduction.

Introduction

One of the best-characterized responses to temperature stress is the induction of heat-shock protein (HSP) expression. All organisms respond to elevated temperatures, as well as many other types of stress, by producing HSPs (Gusev *et al.*, 2005; Kotak *et al.*, 2007; Mayer and Bukau, 2005; Sung *et al.*, 2001; Terasawa *et al.*, 2005). Expression of HSPs is also essential in the acquisition of thermotolerance by plants (Charng *et al.*, 2006; Lee *et al.*, 2005; Miroshnichenko *et al.*, 2005; Queitsch *et al.*, 2000; Sanmiya *et al.*, 2004).

Heat-shock transcription factor (HSF) proteins are present in the cytoplasm as inactive monomers. The heat-shock response begins with the trimerization of an HSF, followed by transport into the nucleus, where it binds to the heat-shock element (HSE) in the promoter region of HSP genes (Baniwal *et al.*, 2004; Hahn *et al.*, 2004; Yamamoto *et al.*, 2005). Binding of an HSF to the HSE activates transcription of HSP genes, and regulation of HSF activity controls the expression of HSP genes. In *Saccharomyces cerevisiae* (Yamamoto *et al.*, 2005) and *Drosophila melanogaster* (Birch-Machin *et al.*, 2005), there are single HSF genes, while the HSFs in higher eukaryotes are encoded by multi-gene families (Baniwal *et al.*, 2004; Scharf *et al.*, 1990). Analysis of the *Arabidopsis thaliana* genome revealed a unique HSF complexity. A total of 21 open reading frames in the *A. thaliana* genome were identified as encoding presumptive HSFs

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(Nover *et al.*, 2001). Many biological processes are regulated by reversible phosphorylation, including the activity of HSF. Phosphorylation of HSF is both constitutive and stress-induced, and involves multiple sites and kinases (Cotto *et al.*, 1996; Guettouche *et al.*, 2005; Kline and Morimoto, 1997; Reindl *et al.*, 1997; Sorger, 1990).

Changes in cytoplasmic Ca^{2+} levels act as a ubiquitous signal in eukaryotic cells. Four classes of Ca^{2+}- or calmodulin (CaM)-dependent protein kinase are involved in amplifying and diversifying the responses to Ca^{2+}-mediated signals (Harper *et al.*, 2004). Holmberg *et al.* (2001) reported that Ca^{2+}/CaM-dependent protein kinase II (CaMKII) phosphorylated Ser230 of HSF1, and overexpression of CaMKII enhanced both the level of *in vivo* Ser230 phosphorylation and transactivation of HSF1. The CaM-binding protein kinases (CBKs) are the least well understood of the CaM target proteins in plants (Zhang and Lu, 2003). Studies indicate that CBKs might mediate responses to light (Pandey and Sopory, 2001) and gravity (Konings, 1995), although there is little direct information about specific functions of CBKs. The data described here indicate that a CaM-binding protein kinase, AtCBK3, acts as a positive regulator of the heat-shock response by phosphorylating AtHSFA1.

Our previous results indicated that intracellular free calcium ion levels ($[Ca^{2+}]_i$) increased during HS of wheat (*Triticum aestivum* L.) seedlings (Liu *et al.*, 2003) and *A. thaliana* suspension cells (Liu *et al.*, 2006). The levels of CaM mRNA and protein increased during HS in the presence of Ca^{2+}, and Ca^{2+} and CaM together regulate expression of HSP genes and synthesis of HSPs (Fan *et al.*, 2000; Liu *et al.*, 2003). Among the nine *AtCaM* genes (*AtCaM1–AtCaM9*), only *AtCaM3* expression increased preceding the increase in expression of the *AtHSP18.2* gene (Liu *et al.*, 2005). The modulation of HSF DNA-binding activity by Ca^{2+}/CaM *in vitro* has been demonstrated previously (Li *et al.*, 2004). Based on our findings, we proposed a pathway for the participation of Ca^{2+}/CaM in HS signal transduction. The specific mechanism by which CaM regulates activation of HSF is not known. Phosphorylation of HSF is performed by a CaM-dependent protein kinase. The results presented demonstrate the involvement of AtCBK3 in HS signal transduction, by promoting phosphorylation of AtHSFA1a and activating the binding activity of HSFs to HSEs. The systematic nomenclature for *A. thaliana* HSF proposed by Nover *et al.* (2001) is used.

Results

AtCBK3 can interact with AtHSFA1a in vivo

In eukaryotic cells, the expression of HSP genes is mediated by HSFs, the terminal components of a signal transduction pathway mediating the response to HS. We proposed previously a pathway for the participation of Ca^{2+}/CaM in HS signal transduction (Liu *et al.*, 2003). To address the questions of how CaM regulates activation of HSF and whether the HSFs can be phosphorylated by CBK, a yeast two-hybrid assay was used to evaluate the interaction between several AtCBKs (AtCBK1–3) and AtHSFA1a. The capacity of the yeast strain AH109 to grow on synthetic medium lacking Trp, Leu, His and adenine, and the β-galactosidase activity were the interaction reporters. In the absence of prey constructs, yeast strains expressing AtHSFA1a grow very slowly on medium lacking Trp, Leu, His and adenine, taking 12 days to grow, and the β-galactosidase reporter was also stimulated to a very low level, requiring 11 h for the filter assay to turn blue. The interaction between AtHSFA1a and AtCBK3 results in quicker growth on selection medium (only 4 days to grow; Figure 1a, image 4), while AtHSFA1a co-transformed with AtCBK1 or AtCBK2 result in no increase in growth rate over AtHSFA1a alone (Figure 1a, images 2 and 3). To confirm the interaction, the yeast strains that grew on medium lacking Trp and Leu were used to carry out filter assay. The interactions between AtHSFA1a and AtCBK3 (Figure 1b, image 4) or AtCBK2 (Figure 1b, image 3) were positive using the X-Gal filter assay, and the color changed to blue in a similar time (approximately 2 h). Thus AtCBK3 can interact with AtHSFA1a but AtCBK1 cannot, and there is the possibility of a weak or transient interaction between AtCBK2 and AtHSFA1a.

The *in planta* interaction between AtHSFA1a and AtCBK3 was confirmed using fluorescence resonance energy transfer (FRET; Lakowicz, 1999; Murata *et al.*, 2000). We examined protein interactions in onion epidermal cells co-expressing AtHSFA1a–CFP (cyan fluorescent protein) and AtCBK3–YFP (yellow fluorescent protein). The method used, FRETN, includes a normalization for the donor and acceptor concentrations (Gordon *et al.*, 1998). Both AtHSFA1a–CFP and AtCBK3–YFP were present in the cytoplasm and the nucleus (Figure 1c, images 1 and 2). Images 3 and 4 in Figure 1(c) are the FRET images before and after correction, respectively. The corrected FRET images show interaction between CFP and YFP, and indicate that AtHSFA1a and AtCBK3 interact *in vivo*. Berney and Danuser (2003) created a FRET model system for the quantitative FRET method. They concluded that 'Donor and acceptor concentration should be of the same order of magnitude, and stable FRET measurements can only be achieved in the range of donor-to-acceptor ratios 0.1–10' (Berney and Danuser, 2003). In our FRET experiment, the fluorescence of YFP ranged from 500 to 600, while that of CFP ranged from 100 to 200, so the fluorescence ratio is within the range 0.2–0.3. Brightness is the product of extinction coefficient and quantum yield (Shaner *et al.*, 2005), and YFP has a greater brightness than CFP (YFP = 51; CFP = 13), hence the ratio is 0.8–1.2 after brightness correction (correction coefficient = 51/13 = 3.92). The *in planta* interaction between AtHSFA1a and AtCBK2 was also tested and shown to be negative (Figure S2).

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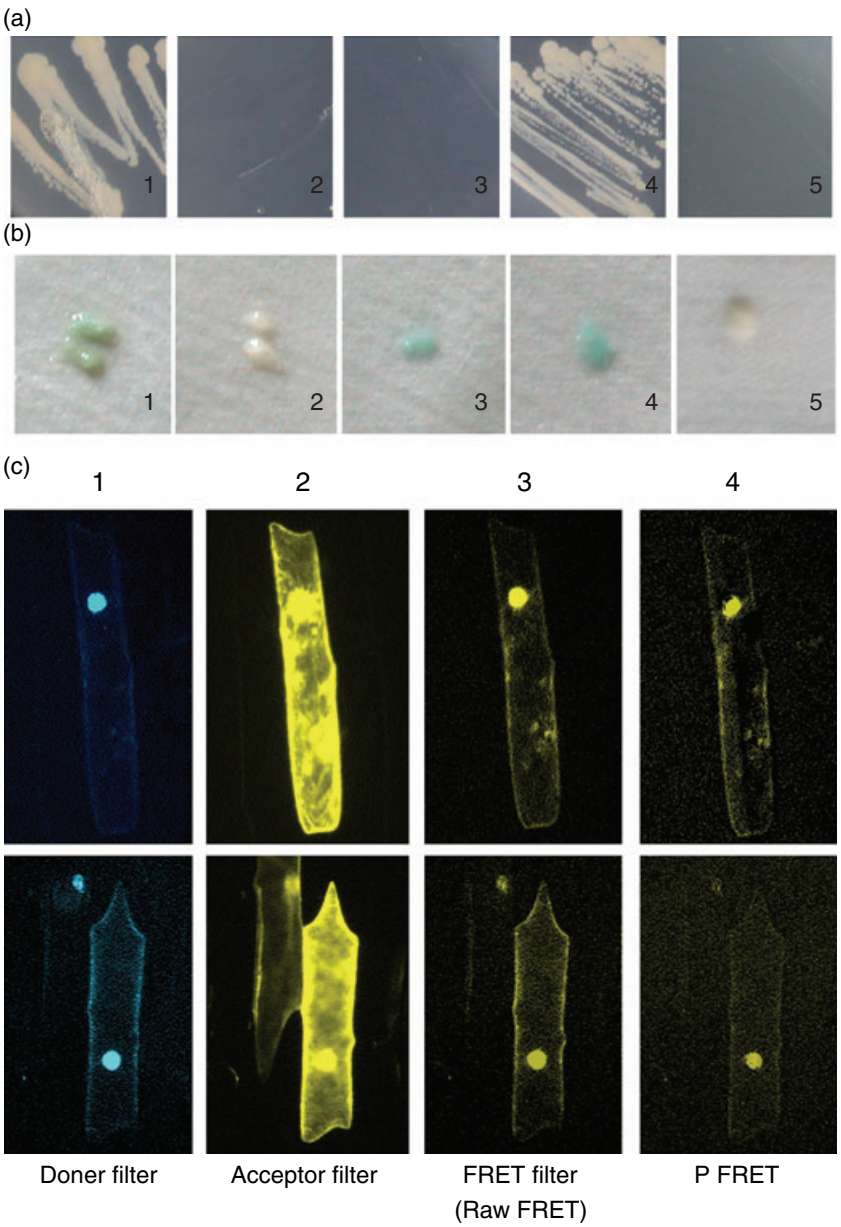

Figure 1. *In vivo* interaction between AtHSFA1a and AtCBK1–3.
(a, b) Growth of the yeast clones on SD medium lacking Trp, Leu, His and adenine (a), and β-galactosidase activity assay (b): (1) positive control; (2) pGADT7-*AtCBK1*/pGBKT7-*AtHSFA1a*; (3) pGADT7-*AtCBK2*/pGBKT7-*AtHSFA1a*; (4) pGADT7-*AtCBK3*/pGBKT7-*AtHSFA1a*; (5) negative control.
(c) Fluorescence intensity and FRET images of *AtHSFA1a–CFP* and *AtCBK3–YFP* (FRETN): (1) fluorescence intensity image taken with the donor filter set; (2) fluorescence intensity image taken with the acceptor filter set; (3) fluorescence intensity image taken with the FRET filter set; (4) corrected FRET image calculated from the equation in Experimental procedures.

Phosphorylation of AtHSF1 in vitro *by AtCBK3*

Because AtHSFA1a and AtCBK3 interact *in vivo*, AtHSFA1a might be a phosphorylation substrate. Both proteins were expressed as GST fusions in yeast (SP-Q01), and purified by GST affinity chromatography (Figure S3). The GST fusions were confirmed on immunoblots using anti-GST antibodies (Figure 2a). The *in vitro* results indicate that AtHSFA1a was specifically phosphorylated by AtCBK3 in the presence of calcium and CaM (Figure 2b, lane 3). The kinase assay was also performed using purified AtCBK2, which could not phosphoralate AtHSFA1a (data not shown).

Expression pattern of AtCBK3

As a step toward determining the function of AtCBK3, the expression pattern of a promoter–GUS fusion was determined in transgenic *A. thaliana* (Figure 3a). The promoter was active throughout the organs and tissues of young seedlings, as well as in flowers and siliques, indicating that expression of *AtCBK3* is not organ- or tissue-specific. Analysis by real-time PCR confirmed above results (Figure 3b). These results suggest the promoter was active throughout the 10–14-day-old seedlings, so we used 10- or 14-day-old seedlings as material in the following experiments.

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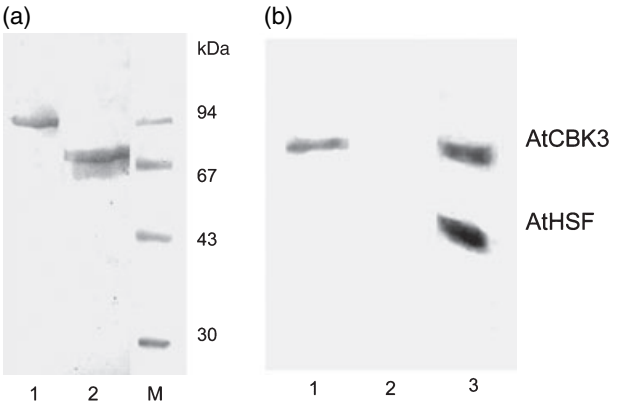

Figure 2. *In vitro* phosphorylation of AtHSFA1a by AtCBK3.
(a) Western blot analyses of the purified chimeric AtCBK3 and AtHSFA1a proteins detected using anti-GST antibodies. Lane 1, AtCBK3–GST; lane 2, AtHSFA1a–GST; M, molecular mass standards.
(b) Autoradiograph of AtHSFA1a–GST phosphorylated *in vitro* by AtCBK3–GST plus γ-^{32}P[ATP]. Lane 1, GST/AtCBK3–GST; lane 2, GST/AtHSFA1a–GST; lane 3, AtCBK3–GST/AtHSFA1a–GST.

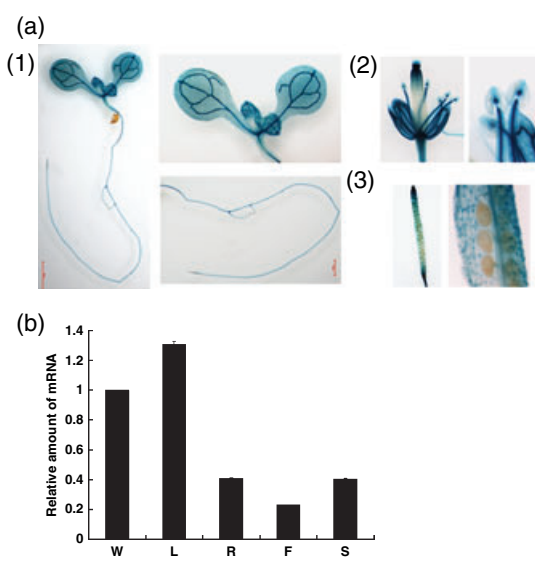

Figure 3. Expression of the *AtCBK3* gene in *A. thaliana*.
(a) Analysis of spatiotemporal expression of the *AtCBK3* gene using a promoter:GUS fusion. Histochemical GUS staining was observed in whole 10-day-old seedlings (1), open flowers (2) and siliques (3).
(b) Analysis of expression of the *AtCBK3* gene in organs of *A. thaliana* by real-time PCR. W, whole seedlings; L, leaves; R, roots; F, flowers; S, siliques.

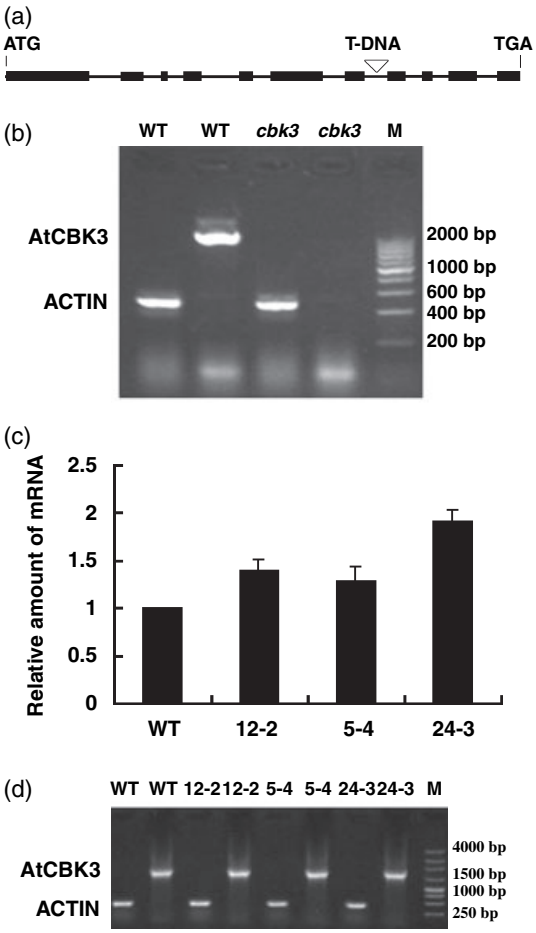

Figure 4. Isolation and complementation of the AtCBK3 T-DNA insertional mutant.
(a) Intron/exon. organization of the *A. thaliana CBK3* gene (coding region), and location of the T-DNA insertion. Solid boxes and lines indicate exons and introns, respectively. The position of the T-DNA insertion is indicated by a triangle.
(b) RT-PCR analysis of the *AtCBK3* transcript in WT and mutant (cbk3) plants. The *actin* expression level was analyzed as a control. The RT-PCR reactions included *actin* and *AtCBK3* whole-sequence primers, respectively. M, molecular mass markers.
(c) Real-time PCR analysis of complemented knockout lines (cbk3/CBK3). Total RNA was isolated from 14-day-old seedlings, converted to cDNA, and then subjected to real-time PCR using gene-specific primers (Table S2). *Actin* was used as a control. The samples from WT seedlings were used as calibrators; the expression level of the samples was set to 1. Each data point is the mean value from biological triplicate determinations.
(d) RT-PCR analysis of *AtCBK3* transcript in WT and complemented knockout lines (12–2, 5–4 and 24–3). The *actin* expression level was analyzed as a control. M, molecular mass markers.

Characterization of an AtCBK3 *T-DNA insertional mutant*

To genetically dissect the function of AtCBK3, we screened T-DNA insertion alleles of the *AtCBK3* gene using lines deposited at the SALK Institute Genomic Analysis Laboratory (http://signal.salk.edu). A single T-DNA insertion line for AtCBK3 was identified (SALK-071004), and designated cbk3. As shown in Figure 4(a), the T-DNA in line cbk3 is inserted into the 7th intron. Analysis by RT-PCR revealed that expression of the *AtCBK3* gene was almost completely suppressed in the mutant compared with the wild-type (WT; Figure 4b). For complementation analysis, we constructed a binary vector containing the whole coding region of the *AtCBK3* gene under the control of the 35S promoter (pCAMBIA1300-*35S:AtCBK3)*. Real-time quantitative PCR

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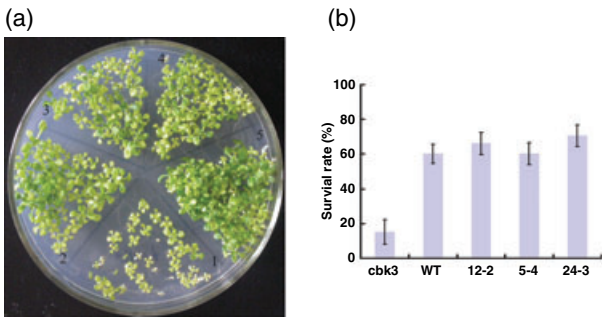

Figure 5. The effects of *AtCBK3* knockout and complemented knockout on the basal thermotolerance (a) and survival rate (b) of *A. thaliana* seedlings.
(a) Basal thermotolerance of *A. thaliana* seedlings: (1) cbk3; (2) WT; (3) cbk3/CBK3 12–2; (4) cbk3/CBK3 5–4; (5) cbk3/CBK3 24–3. Ten-day-old seedlings grown at 22°C were shifted to 45°C for 30 min, and then returned to 22°C. Plants were photographed 7 days after return to 22°C.
(b) Comparison of survival rate among mutant cbk3, WT and complemented knockout lines 12–2, 5–4 and 24–3. The growths condition and heat treatment were the same as in (a). Each data point is the mean value from five independent experiments (50 seedlings per experiment).

(real-time PCR) was used to study the expression of transgenic plants containing the complementation construct. The results show that most transgenic plants containing the construct expressed the *AtCBK3* mRNA at a level comparable with that of WT plants. The complemented transgenic lines cbk3/AtCBK3 12–2 and 5–4 expressed a comparable level of *AtCBK3* mRNA, while line cbk3/AtCBK3 24–3 showed about twofold greater expression of the *AtCBK3* transcript (Figure 4c). Analysis by RT-PCR confirmed that expression of the *AtCBK3* gene in the complemented mutant lines 12–2, 5–4 and 24–3 was rescued (Figure 4d).

No significant phenotypic changes were observed in line cbk3 compared with WT plants. The cbk3 mutant plants were then tested for differences in their responses to heat stress. Seedlings of 10-day-old cbk3 mutant, WT and complemented mutant plants were subjected to 45°C for 30 min, then returned to 22°C. The cbk3 mutant plants were impaired in basal thermotolerance (Figure 5a), and exhibited a significant delay in growth after heat treatment. Expression of *AtCBK3* in line cbk3 was able to complement the heat sensitivity. Of ten homozygous lines obtained, six exhibited increased or similar thermotolerance compared to WT, but much higher thermotolerance than the mutant. The thermotolerance of the independent lines cbk3/AtCBK3 12–2 and 5–4 was the same as that of the WT, and line cbk3/AtCBK3 24–3 grew better than the WT after heat stress. The rescue of thermotolerance in the complemented mutant lines was confirmed by calculation of the survival rate. The 10-day-old seedlings were subjected to a 45°C HS for 30 min followed by recovery at 22°C for 7 days, and were then used to calculate the survival rate. The complemented mutant lines 12–2, 5–4 and 24–3 all exhibited higher survival rates after heat stress than mutant line cbk3, similar to the WT (Figure 5b).

Construction and phenotypic analysis of transgenic lines

Transgenic *AtCBK3*-overexpressing plants were prepared using the binary vectors pCAMBIA1300-*35S:AtCBK3* or pCAMBIA1300-*35S:AtCBK3-GFP*. Twenty-eight independent T_3 lines with a single copy of *35S:AtCBK*, together with 23 independent T_3 transgenic lines with a single copy of *35S:AtCBK3-GFP*, were generated. The basal thermotolerance of the transgenic plants was tested and compared with that of WT or control plants transformed with the empty pCAMBIA1300 vector (plants 1300). Homozygous *AtCBK3* transgenic plants, and WT or control plants 1300, were plated and grown for 10 days. All seedlings were exposed to 45°C for 45 min, and then returned to 22°C. Almost half (46%) of the *35S:AtCBK3* and 61% of the *35S:AtCBK3–GFP* lines exhibited increased thermotolerance compared with WT or control plants 1300.

To ensure that the phenotypic changes observed were caused by overexpression of the *AtCBK3* transcript, total RNA was isolated from seedlings of four *35S:AtCBK3* transgenic lines (11–6, 60–2, 34–3, 76–2) and three *35S:AtCBK3–GFP* transgenic lines (22–2, 5–6, 3–2) showing higher basal thermotolerance. Using real-time PCR analysis, we observed clear overexpression of the *AtCBK3* gene in all transgenic lines tested, especially *35S:AtCBK3* line 34–3 and *35S:AtCBK3–GFP* line 5–6. Line 34–3 had a 12-fold increase, while line 5–6 showed a 7-fold increase in *AtCBK3* transcript levels compared to WT (Figure 6a,d). The basal thermotolerance of the 10-day-old transgenic seedlings, especially *35S:AtCBK3* line 34–3 and *35S:AtCBK3–GFP* line 5–6, was increased compared with WT or control plants 1300. Most of the WT or control plants 1300 died after incubation at 45°C for 45 min. The *AtCBK3*-overexpressing plants survived this lethal stress. They exhibited a delay in growth after the heat stress, but turned green and resumed growth after 7 days of recovery at 22°C (Figure 6b,e). The survival rate of seedlings after the heat stress was calculated. The survival rate of *AtCBK3*-overexpressing transgenic lines including *35S:AtCBK3* lines and *35S:AtCBK3–GFP* lines was higher than that of WT or control plants 1300 (Figure 6c,f). These results confirm the role of the *AtCBK3* gene in improving the basal thermotolerance of *A. thaliana* seedlings.

Regulation of AtCBK3 on the activity of HSF and expression of HSP genes during HS

Heat-shock proteins are induced by HS, and play an important role in acquisition of thermotolerance by plants (Charng *et al.*, 2006; Lee *et al.*, 2005; Miroshnichenko *et al.*, 2005; Queitsch *et al.*, 2000; Sanmiya *et al.*, 2004). To understand whether expression of the *AtCBK3* gene affects the expression of HSP genes, which subsequently affect thermotolerance of *A. thaliana* seedlings, a gel mobility shift assay was

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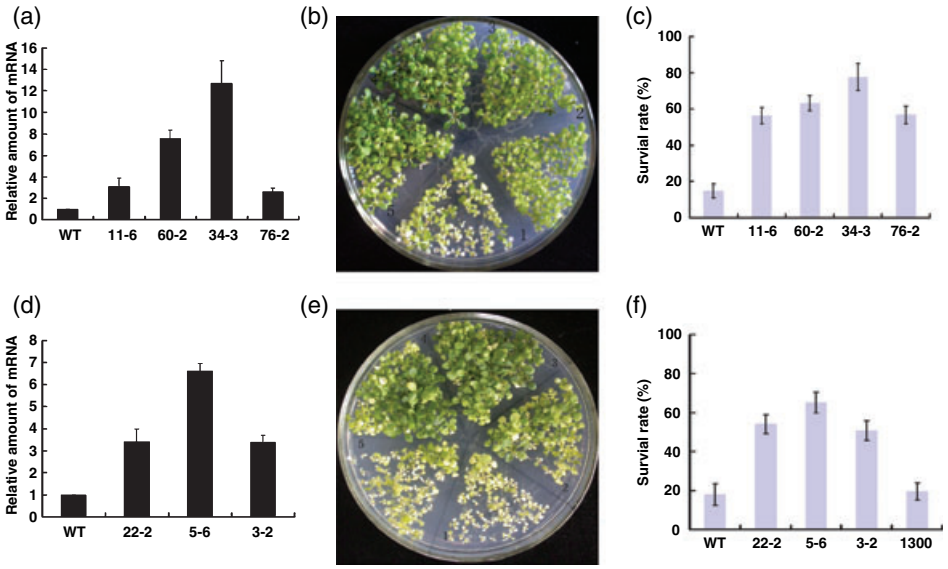

Figure 6. Overexpression of *AtCBK3* improves basal thermotolerance of *A. thaliana* seedlings.
(a, d) Real-time PCR analysis of *AtCBK3* expression in various *35S:AtCBK3* (a) or *35S:AtCBK3–GFP* (s) plants. The real-time PCR conditions were the same as described in Figure 4(c).
(b, e) Overexpression of *AtCBK3* in various *35S:AtCBK3* (b) or *35S:AtCBK3–GFP* (e) plants improves the basal thermotolerance of transgenic seedlings. Ten-day-old seedlings of WT or transgenic lines grown at 22°C were shifted to 45°C for 45 min, and then returned to 22°C. Seedlings were photographed 7 days after return to 22°C.
(b) (1) WT; (2)–(5) transgenic lines 11–6, 60–2, 34–3 and 76–2. (e) (1) WT; (2)–(4) transgenic lines 22–2, 5–6 and 3–2; (5) empty vector control plant 1300.
(c, f) Survival rate of WT, control plants 1300 and various *35S:AtCBK3* (c) or *35S:AtCBK3–GFP* (f) transgenic lines. The growth conditions and heat treatment were the same as in (b) and (e). Each data point is the mean value from five independent experiments (50 seedlings per experiment).

used to analyze the difference in binding activity of HSFs to HSEs in the cbk3 mutant plants, WT and three independent *AtCBK3*-overexpressing lines (34–3, 76–2, 11–6) after HS. Samples of whole-cell extracts from 10-day-old seedlings were heat-shocked at 37°C for 30 min, and a gel mobility shift assay was performed. Figure 7(a) shows that the binding activity of HSF was weaker in the cbk3 mutant line, but stronger in the three *AtCBK3*-overexpressing lines (34–3, 76–2, 11–6) than in WT plants.

Furthermore, we analyzed the expression of *AtHSP18.2*, *AtHSP25.3*, *AtDjA2* (*HSP40*) and *AtHSP83* in the cbk3 mutant plants, complemented mutant plants (line 12–2), WT and three independent *AtCBK3*-overexpressing lines (34–3, 76–2, 11–6) after incubation at 37°C for 1 h using real-time PCR. After HS, expression of all the HSP genes tested in the cbk3 mutant seedlings was only half of that in the WT seedlings. Overexpression of *AtCBK3* in the cbk3 mutant was able to reverse the inhibition of expression of HSP genes. The mRNA level of HSP genes tested in the complemented line cbk3/CBK3 12–2 was comparable to that in the WT plants. Expression of the four HSP genes in the various *AtCBK3*-overexpressing lines was higher than that in the WT plants. In particular, the expression of *AtHSP18.2*, *AtHSP25.3* and *AtHSP83* in line 34–3 was 2–3-fold higher than that in the WT plants (Figure 7b–e). The effect of AtCBK3 on the synthesis of HSPs was also tested. Both 14-day-old WT seedlings and independent *AtCBK3*-overexpressing lines were treated at 37°C for 2 h or maintained at 22°C. Protein samples were separated by SDS–PAGE, and analyzed by immunoblotting. The HSP18.2 was detected using anti-Arabidopsis HSP 18.2 antiserum. The chloroplast-localized sHSP (ClsHSP, small HSP) AtHSP25.3 was detected using anti-tomato ClsHSP antiserum. The results show that neither AtHSP25.3 nor AtHSP18.2 were expressed at 22°C, but were induced by HS. The accumulation of both AtHSP25.3 and AtHSP18.2 proteins in the *AtCBK3*-overexpressing lines (11–6, 34–3, 76–2) was higher than that in WT under HS conditions (Figure 8). Thus, changes in the expression of HSP genes in both cbk3 mutant plants and *AtCBK3*-overexpressing plants are linked to the change in thermotolerance.

Discussion

Interaction of the CaM-binding protein kinases with HSFs

Several Ca^{2+}/CaM-dependent protein kinases are present in animal systems, and there are numerous reports of their functional significance. However, the CBKs are the least well understood of the CaM target proteins in plants (Zhang and Lu, 2003). Watillon *et al.* (1995) isolated the first of the plant CBKs, CB1, from apple, followed by the isolation of MCK from maize (Lu *et al.*, 1996), OsCBK from rice (Zhang *et al.*, 2002), AtCBK1, 2 and 3 from *A. thaliana* (Wang *et al.*, 2004; Xie *et al.*, 2002; Zhang and Lu, 2003), and NtCaMK1 and

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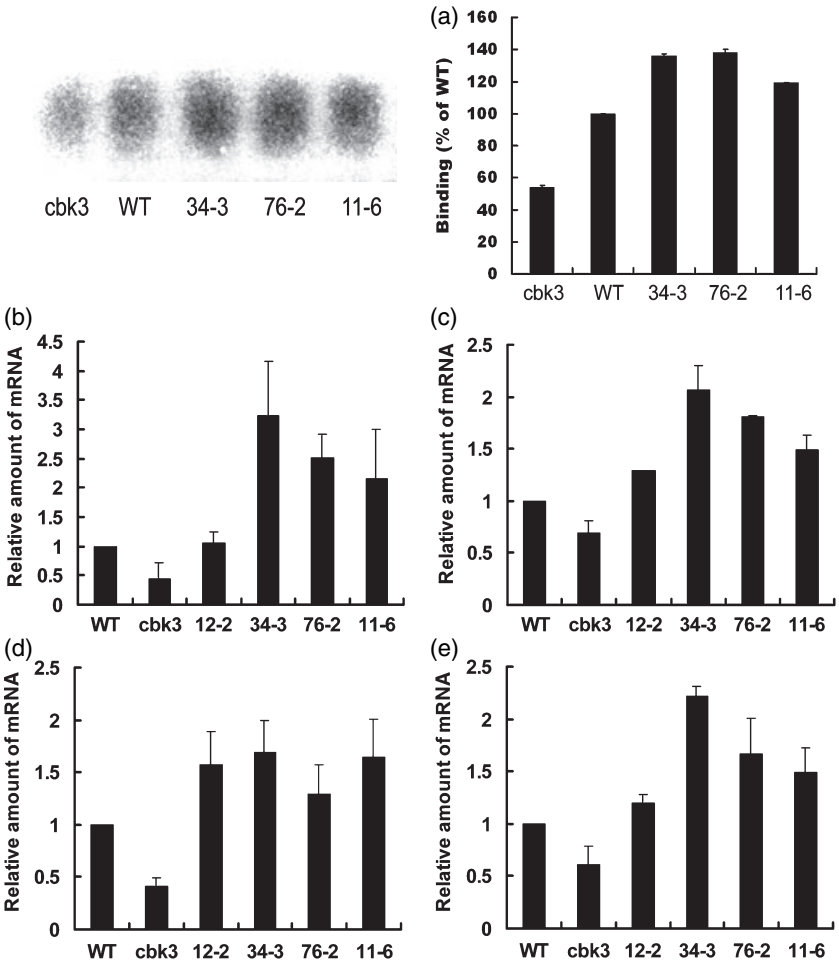

Figure 7. Binding activity of HSFs to HSEs, and expression of HSPs in WT, cbk3 and *AtCBK3*-overexpressing seedlings.
(a) Binding activity of HSFs to HSEs. Whole-cell extracts weres obtained from 10-day-old cbk3 mutant, WT and *AtCBK3*-overexpressing seedlings (lines 34–3, 76–2, 11–6) and treated at 37°C for 30 min. Samples of whole-cell extracts with the same protein content (30 μg) were used for electrophoresis and binding assays. Images (left) and quantification of the shifted bands (right) are shown.
(b–e) Real-time PCR analyses of expression of HSP genes in WT, cbk3, cbk3/CBK3 line 12–2 and *AtCBK3*-overexpressing lines 34–3, 76–2 and 11–6. (b) *AtHSP18.2*; (c) *AtHSP25.3*; (d) *AtDjA2*; (e) *AtHSP83*. The 14-day-old seedlings were heat-shocked at 37°C for 1 h. After HS, RNA was isolated and used for real-time PCR analyses as described in Figure 4(c).

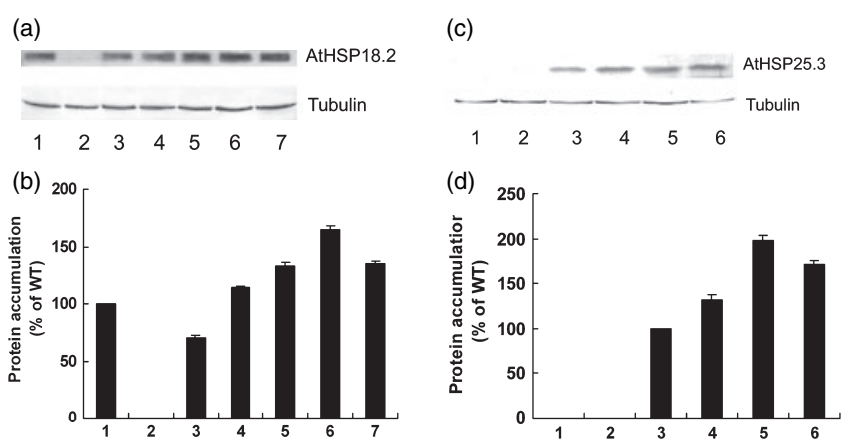

Figure 8. Accumulation of HSP proteins in WT, cbk3 and *AtCBK3*-overexpressing seedlings.
The 14-day-old seedlings were treated at 37°C for 2 h. After HS, total protein samples were extracted, and samples corresponding to 30 mg of protein were separated by SDS–PAGE, and analyzed by immunoblotting. Tubulin was used as the quantification control.
(a) Images and (b) quantification of shifted bands for the AtHSP18.2 protein (detected using anti-Arabidopsis HSP18.2 antiserum). (1) WT after HS; (2) WT under non-HS conditions; (3) mutant cbk3 after HS; (4) cbk3/CBK3 line 12–2 after HS; (5)–(7) *AtCBK3*-overexpressing plants 11–6, 34–3 and 76–2 after HS.
(c) Images and (d) quantification of shifted bands for the AtHSP25.3 protein (detected using anti-tomato ClsHSP antiserum). (1) WT under non-HS conditions; (2) *AtCBK3*-overexpressing line 34–3 under non-HS conditions; (3) WT after HS; (4)–(6) *AtCBK3*-overexpressing lines 11–6, 34–3 and 76–2 after HS.

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NtCBK1 from tobacco (Hua *et al.*, 2004; Ma *et al.*, 2004). The results of studies during the past decade have indicated that CBKs are involved in responses to environmental changes. Red light downregulates the expression of *Zea mays* ZmCCaMK (calcium/calmodulin-dependent protein kinase), suggesting that this CBK is involved in a light-dependent signal transduction pathway (Pandey and Sopory, 2001). Cold and salt stress upregulate the expression of pea PsCCaMK, and the expression of NtCaMK1 is also upregulated by salt stress (Pandey *et al.*, 2002; Zhang and Lu, 2003). It was also reported that Ser230 of human HSF1 is positioned within a consensus site for CaMKII, and that overexpression of CaMKII increased the levels of both *in vivo* Ser230 phosphorylation and transactivation of HSF1 (Holmberg *et al.*, 2001). Thus, CaMKII may be a positive regulator of human HSF1.

In eukaryotes, expression of HSP genes is mediated by HSFs (Baniwal *et al.*, 2004; Nover *et al.*, 2001; Wunderlich *et al.*, 2003). The HSFs are the terminal components of a signal transduction pathway mediating activation of genes responsive to both HS and a large number of chemical stressors. The binding of HSFs to HSEs in HSP promoters activates transcription of multiple HSP genes. Phosphorylation has been also proposed to play an important role in activation and inactivation of HSFs (Dai *et al.*, 2000; Guettouche *et al.*, 2005; Hashikawa and Sakurai, 2004; Schöffl *et al.*, 1998; Wang *et al.*, 2006). HsfA1a was defined as a master regulator of the heat-shock response in tomato (Mishra *et al.*, 2002). Suppression of AtHSFA1a activity impairs both basal and acquired thermotolerance of *A. thaliana* (Wunderlich *et al.*, 2003). AtHSFA1a is undoubtedly one of the master regulators for the expression of HSPs and thermotolerance, although regulation of the heat-shock response by AtHSFA2 was also recently documented (Charng *et al.*, 2007; Li *et al.*, 2005; Nishizawa *et al.*, 2006). We chose AtHSFA1a as a target gene for AtCBK3 in this experiment. We report that AtCBK3 can interact with AtHSFA1a *in vivo*, and that AtHSFA1a can be phosphorylated *in vitro* by AtCBK3 in the presence of Ca^{2+} and CaM (Figures 1 and 2).

Wang *et al.* (2004) reported that AtCBK3 activity could be stimulated by CaM. In the presence of both Ca^{2+} and CaM, the maximal activity was approximately 10-fold higher than the basal activity in the presence of Ca^{2+} alone. In our experiment, addition of CaM caused only a 2–3-fold increase in the activity of AtCBK3 (Figure S4). This may be due to the different substrates for phosphorylation by AtCBK3. The substrates used by Wang *et al.* were histone IIIS and syntide-2, whereas the substrate used in our experiment was the AtHSFA1a–GST fusion protein. CaM is able to affect the phosphorylation of different substrates by AtCBK3, but the extent of phosphorylation differs due to the different molecular mass and structure of different substrates.

The effects of AtCBK3 on thermotolerance in A. thaliana

Downregulating *AtCBK3* expression by T-DNA insertional mutagenesis impaired thermotolerance. Multiple independent lines of complementary transformants were able to rescue the lower thermotolerance of the mutant cbk3 (Figures 4 and 5), whereas multiple independent transformants that overexpress *AtCBK3* displayed higher thermotolerance (Figure 6). The change in thermotolerance is related to the change in expression of the *AtCBK3* gene in a dose-dependent fashion.

Cells synthesize HSPs in response to elevated temperature, as well as other environmental stresses (Kilstrup *et al.*, 1997; Neven *et al.*, 1992). HSPs function as molecular chaperones, which are essential for cellular processes. Under stress conditions, the synthesis of molecular chaperones allows cellular proteins to avoid and/or recover from stress-induced protein aggregation (Hartl and Hayer-Hartl, 2002; Mayer and Bukau, 2005; Miernyk, 1999; Nollen and Morimoto, 2002). The functional analysis for individual HSPs has been documented. HSP101 plays a critical role in thermotolerance (Queitsch *et al.*, 2000; Yang *et al.*, 2006), and the role of small HSPs in increasing thermotolerance has been reported (Charng *et al.*, 2006; Li *et al.*, 2007; Miroshnichenko *et al.*, 2005; Sanmiya *et al.*, 2004). Regulation of activity of HSFs is a central mechanism of transcriptional regulation for HSP gene expression. The HSFs are activated by HS or other stresses, leading to specific binding to HSEs. Binding of HSFs to HSEs resulted in transcription of multiple HSP genes and increase of thermotolerance (Baniwal *et al.*, 2004; Kotak *et al.*, 2007; Nover *et al.*, 2001; Schöffl *et al.*, 1998). We first measured binding activity of HSFs to HSEs in various lines in order to assess the role of AtCBK3 in HS signal transduction. The results reported here demonstrate that AtCBK3 affects the binding activity of HSFs to HSEs – there is higher binding activity in the lines with higher expression of *AtCBK3* (Figure 7a). AtCBK3 also regulates the expression of HSP genes – downregulation of *AtCBK3* expression leads to lower expression of HSP genes, while overexpression of *AtCBK3* results in an increase in expression of HSP genes in *A. thaliana* seedlings after HS (Figure 7b–e). Furthermore, overexpression of *AtCBK3* increased synthesis of the AtHSP18.2 and AtHSP25.3 proteins in *A. thaliana* seedlings (Figure 8). Together, these results support the proposal that AtCBK3 modulates the expression of HSP genes by regulating the phosphorylation status and thus the activity of AtHSFA1a. Therefore, AtCBK3 is involved in HS signal transduction as a positive regulator of HSF activity.

In our experiment, lines 76–2 and 34–3 showed similar increases in HSF binding to HSE (Figure 7a), but line 76–2 showed lower levels of HSP mRNA (Figure 7b–e), protein accumulation (Figure 8) and survival rate (Figure 6c) than line 34–3. The reason for this may be that the cDNA of AtCBK3 was inserted at different locations in the genome in

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lines 76–2 and 34–3, based on the results of Southern blotting of AtCBK3 (Figure S5). Many modulations, including transcriptional, post-transcriptional, translational, post-translational and physiological controls, are involved in the biological processes linking activation of HSF, expression of HSPs and thermotolerance. Genes, proteins and enzymes that participate in the regulatory events may be affected differently due to the different location of insertion, causing the different levels of HSP mRNA and protein and lower survival rate in line 76–2.

AtCBK3 is an important member in the Ca^{2+}/CaM pathway of HS signal transduction

Calcium is a universal second messenger, and acts as a mediator of stimulus–response coupling to regulate diverse cellular functions (Harper *et al.*, 2004; McCormack *et al.*, 2005; Reddy and Reddy, 2004). In plant cells, stimuli such as water stress, cold, wind, touch and wounding all cause an increase in $[Ca^{2+}]_i$, and Ca^{2+} has been proposed as an important upstream component in HS signal transduction. 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). 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.

As a mediator of Ca^{2+} signals, CaM is activated by binding Ca^{2+}, inducing a cascade of regulatory events (McCormack *et al.*, 2005; Yang and Poovaiah, 2003). Accumulation of CaM in maize seedlings was induced by HS in the presence of Ca^{2+}(Gong *et al.*, 1997). The expression of CaM or CaM-related genes is induced by many environmental stresses such as wind, touch, cold shock, pathogen attack and wounding of plants (Braam, 1992; Heo *et al.*, 1999; van der Luit *et al.*, 1999; McCormack *et al.*, 2005). Previous results have indicated that $[Ca^{2+}]_i$ increased in wheat seedlings during HS, the levels of CaM mRNA and protein increased during HS in the presence of Ca^{2+}, and that Ca^{2+} and CaM promoted expression of HSP genes in both wheat and *A. thaliana* (Fan *et al.*, 2000; Liu *et al.*, 2003, 2005). Furthermore, the involvement of Ca^{2+}/CaM in activating HSF for binding to HSE *in vitro* in maize has been reported (Li *et al.*, 2004). A new pathway of HS signal transduction, the Ca^{2+}/CaM pathway, was proposed by Liu *et al.* (2003). The HS signals are perceived by an as yet unidentified receptor, and receptor activation is closely followed by an increase in $[Ca^{2+}]_i$ through opening of Ca^{2+} channels in the plasma membrane or an intracellular Ca^{2+} storage membrane. We have provided primary evidence for the involvement of IP_3/PLC (inositol-1,4,5-triphosphate/phospholipase C) in increasing cytoplasmic $[Ca^{2+}]_i$ and HS signal transduction in *A. thaliana* (Liu *et al.*, 2006). The elevated level of cytoplasmic $[Ca^{2+}]_i$ then directly activates CaM, and also promotes its expression and accumulation. Activated CaM promotes the DNA-binding activity of HSF, and initiates transcription and translation of the HSP genes (Liu *et al.*, 2003). The regulation of HSP expression and thermotolerance by a CaM-binding protein phosphatase (PP7) has been reported (Liu *et al.*, 2007). These results support our model for the involvement of Ca^{2+}/CaM in HS signal transduction. Here we further elaborate the signal transduction pathway with the proposal that AtCBK3 is involved as a positive regulator of AtHSFA1a function. During HS, CaM regulates the activity of AtCBK3 or a CaM-binding protein phosphatase, promoting activation of AtHSFA1a by phosphorylation/dephosphorylation, and expression of HSP genes (Figure 9). AtCBK3 is the key connection between CaM and HSF in the Ca^{2+}/CaM pathway of HS signal transduction.

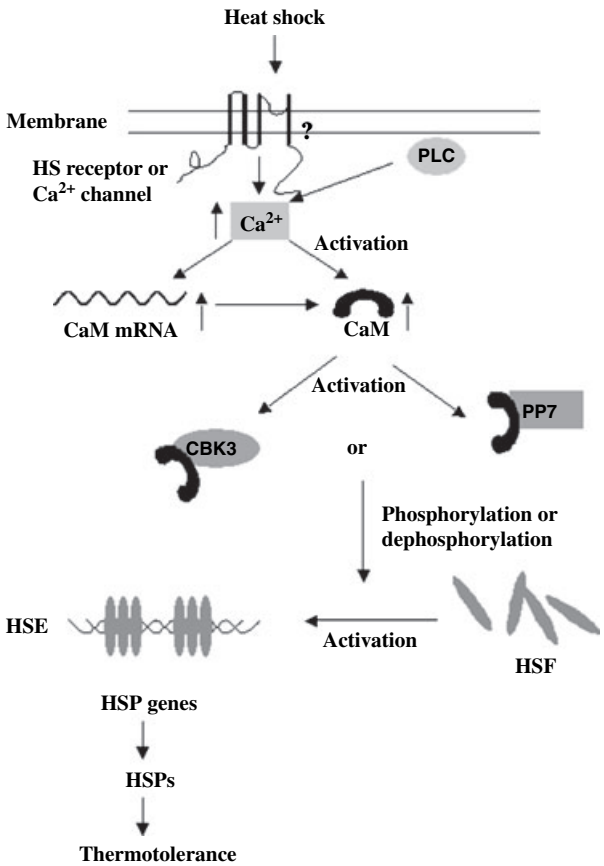

Figure 9. Model for the involvement of CBK protein in the Ca^{2+}/CaM pathway in HS signal transduction. The question mark indicates that heat-shock receptor and Ca^{2+} channels have not yet been resolved during heat-shock signal transduction pathway.

In summary, we believe that AtHSFA1a is an *in vivo* target of AtCBK3, and that phosphorylation of AtHSFA1a by AtCBK3 influences expression of HSPs and thermotolerance of *A. thaliana* seedlings. Thus AtCBK3 is an important component in HS signal transduction downstream of CaM in the Ca^{2+}/CaM pathway.

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Experimental procedures

Yeast two-hybrid analysis

The coding regions of *AtHSFA1a*, *AtCBK1*, *AtCBK2* and *AtCBK3* were amplified using the primer pairs listed in Table S1, then *AtHSFA1a* was ligated with *Nco*I/*Pst*I-digested pGBKT7 (Clontech, http://www.clontech.com/), and *AtCBK1*, *AtCBK2* and *AtCBK3* were ligated with *Bam*HI/*Xho*I-, *Xho*I- and *Sma*I/*Bam*HI-digested pGADT7 (Clontech), respectively.

The yeast two-hybrid constructs pGADT7-*AtCBK1/AtCBK2/AtCBK3* and pGBKT7-*AtHSFA1a* were introduced into the yeast strain AH109 (Clontech) using the lithium acetate method. Cells were plated onto selective medium lacking Trp and Leu, then putative transformants were transferred to medium lacking Trp, Leu, His and adenine. The activity of β-galactosidase was monitored using the substrate 5-bromo-4-chloro-β-D-galactoside (X-Gal) in a colony-lift filter assay (Breeden and Nasmyth, 1985).

FRET analysis

Two constructs were created for FRET measurements. The coding region of *AtHSFA1a* was amplified by PCR using the forward primer 5′-CATGCCATGGGTTTTGTAAATTTCAAA-3′ and the reverse primer 5′-CATGCCATGGCGTGTTCTGTTTCTGA-3′. The coding region of *AtCBK3* was amplified by PCR using the forward primer 5′-CATGCCATGGGGATGTGTCATG-3′ and the reverse primer 5′-CATGCCATGGCAGCTTTTTGTAAGGT-3′. The PCR products were digested with *Nco*I (site underlined) and cloned into the plasmid pAVA-CFP or pAVA-YFP.

pAVA-*AtHSF1-CFP* and pAVA-*AtCBK3-YFP* were co-transformed into onion epidermis cells by microprojectile bombardment and co-expressed. We also transformed pAVA-*AtHSFA1a-CFP* or pAVA-*AtCBK3-YFP* into onion epidermis cells separately. The onion cells were plated onto half-strength MS agar plates and incubated at 22°C for 20 h, then observed by laser scanning confocal microscopy.

Three filter sets (Sutter Instrument Company, http://www.sutter.com) were used for the FRET measurements: (i) the donor filter set, consisting of an ET430/24x excitation filter and an ET470/24m emission filter; (ii) the acceptor filter set, consisting of an ET500/20x excitation filter and an ET535/30m emission filter; (iii) the FRET filter set, consisting of an ET430/24x excitation filter and an ET535/30m emission filter. The p-FRET (processed FRET) signal was calculated using the software MetaMorph 6.2 (Metamorph, http://www.moleculardevices.com/pages/software/metamorph.html) based on the equation: p-FRET = Raw FRET − A*accepter − B*donor, where coefficient A is the mean threshold intensity of acceptor images from the FRET filter set/mean threshold intensity of acceptor images from the acceptor filter (Figure S1), and coefficient B is the mean threshold intensity of donor images from the FRET filter set/mean threshold intensity of donor images from the donor filter set (Figure S1).

Heterologous expression of AtCBK3 and AtHSFA1a proteins

To express AtCBK3 and AtHSFA1a in yeast cells as chimera with glutathione-*S*-transferase (GST), the AtCBK3 coding region was amplified by PCR from AtCBK3 cDNA using the forward primer 5′-CGGGAT*CCATGG*GGATCTGTCATG-3′ and the reverse primer 5′-ACAT*GCATGC*CTAAGCTTTTTGTAAG-3′. The PCR product was restriction digested, then ligated with the yeast expression vector pESP-2 (Stratagene, http://www.stratagene.com/) previously digested with *Bam*HI and *Sph*I. The AtHSFA1a coding region was amplified by PCR from the AtHSFA1a cDNA with using the forward primer 5′-GA*AGATCT*TATGTTTGTAAATTTC-3′ and the reverse primer 5′-GA*AGATCT*CTAGTGTTCTGTTTC-3′) primers. The PCR product was restriction-digested, then ligated with pESP-2 previously digested with *Bgl*II. The expression constructs were transformed into *Schizosaccharomyces pombe* SP-Q01 (Stratagene), and positive colonies were identified by their ability to grow on Edinburgh minimal medium (Stratagene) supplemented with thiamine.

The GST fusion proteins were induced by growing the cells in Edinburgh minimal medium without thiamine, then the yeast cells were harvested, lysed and clarified by centrifugation at 12 000 **g** for 20 min. Clarified supernatants were applied to GST affinity columns (Stratagene), which were washed extensively with 50 mM Tris/HCl, pH 7.3, 100 mM NaCl, 0.1% Tween-20 and 10% glycerol. Specifically bound proteins were eluted with 50 mM Tris/HCl, pH 8.0, containing 10 mM reduced glutathione. The free glutathione was removed using a Centriprep concentrator (Millipore, http://www.millipore.com), and the buffer was exchanged for storage buffer (50 mM Tris/HCl, pH 7.6, 50 mM KCl, 2 mM DTT, 10% glycerol). All procedures were performed at 4°C, unless stated otherwise.

In vitro *kinase assay*

One microgram of recombinant AtCBK3 or GST (control), 5 μg of AtHSFA1a and 1 μM, CaM were incubated in 50 μl of reaction buffer (50 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM ATP). Phosphorylation was initiated by adding 25 μCi of [γ-^{32}P]-ATP, followed by incubation at 30°C for 30 min. Reactions were terminated by adding a one-fifth volume of 5× SDS–PAGE sample buffer. Proteins were separated by 10% SDS–PAGE, and the gels were vacuum-dried for autoradiography.

Plant materials and growth conditions

Seeds of *A. thaliana* (ecotype Columbia) were surface-sterilized, plated on MS medium containing 1.0% w/v sucrose and 0.8% agar, and kept at 4°C for 3 days. Plants were then grown to the flowering stage in lit growth chambers at 22°C and approximately 100 μmol photons m^{-2} sec^{-1} on a 16 h day/night cycle.

A T-DNA insertion line for AtCBK3 (SALK-071004) was obtained from the SALK Institute Genomic Analysis Laboratory. Plants homozygous for the cbk3 insertion were used for further analysis.

Assays of basal thermotolerance were conducted essentially as described by Larkindale *et al.* (2005). Plates were exposed to a 45°C HS for 30 or 45 min, and then allowed to recover at 22°C for 7 days. Seedlings were photographed to record viability. The survival rate was determined as follows. The seedlings were grown under the same conditions and with the same heat treatment as above. After 7 days of recovery at 22°C, the survival of seedlings was monitored. The seedlings that were still green and producing new leaves were scored as surviving. The survival rate is the ratio of surviving seedlings to total seedlings planted. Each data point is the mean value from five independent experiments (50 seedlings per experiment).

Analysis of AtCBK3 promoter–GUS expression in transgenic plants

The *AtCBK3* promoter was isolated by PCR using genomic DNA as the template and the forward primer 5′-AA*CTGCAG*TCAAATCATTGCCTTCCAAC-3′ and reverse primer 5′-GC*TCTAGA*GAATTGTCAATTCGAAACGAAAAG-3′. The 1838 bp PCR fragment was ligated with *Pst*I/*Xba*I-digested pCAMBIA1300 (CAMBIA, http://www.cambia.org). Ecotype Columbia plants were transformed by floral dip (Clough and Bent, 1998); transformants selected on medium containing 25 μg ml^{-1} hygromycin.

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Histochemical staining for GUS expression was performed according to the method described by Jefferson *et al.* (1987). Plants were incubated in 50 mM $NaPO_4$, pH 7.0, containing 10 mg ml^{-1} X-Gluc and 0.02% v/v Triton X-100 at 22°C for 16 h. After staining, plants were washed with 70% ethanol for 1 h, followed by 100% ethanol for 3 h at room temperature.

Analysis of AtCBK3 *expression by RT-PCR*

Transcript abundance in wild-type, T-DNA insertion mutant plants and complemented mutant lines was determined by RT-PCR. Denatured total RNA (1 μg) plus the forward primer 5′-TCCCCCGGGGATCTGTCATGGAAAACC-3′ and the reverse primer 5′-CGGGATCCGAATCTTTGACTCAGAAGC-3′ were used with the One Step RNA RT-PCR kit (Takara, http://www.takara.com). Reverse transcription proceeded for 30 min at 50°C, followed by 94°C for 2 min, then 30 cycles of PCR at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. The *actin* transcript was used as the internal control, with the forward primer 5′-AGGCACCTCTTAACCCTAAAGC-3′ and the reverse primer 5′-GGACAACGGAATCTCTCAGC-3′.

Construction of transgenic lines

The *AtCBK3* coding region was amplified by PCR from *AtCBK3* cDNA using the forward primer 5′-GCTCTAGAAACAATGGGG-ATCTGTCATG-3′ and the reverse primer 5′-GGACTAGTCTAAG-CTTTTTGTAAG-3′. The PCR product was cloned into the binary vector pCAMBIA1300 digested with *Xba*I and *Spe*I. For construction of the AtCBK3–GFP fusion, the *AtCBK3* cDNA was used as the template and amplified by PCR using the forward primer 5′-GCTCTAGATTCAATGGGGATCTGTCATG-3′ and the reverse primer 5′-CGGGATCCCAGCTTTTTGTAAGGTC-3′. The fragment was cloned into the binary vector pCAMBIA1300-*35S:GFP* using *Xba*I and *Bam*HI sites. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. The pCAMBIA1300-*35S:AtCBK3* vector was introduced into both the cbk3 mutant and WT plants, and the pCAMBIA1300-*35S:AtCBK3-GFP* vector was introduced into WT plants, by the floral dip method (Clough and Bent, 1998). Transformants were selected on MS medium containing 25 μg ml^{-1} hygromycin; homozygous transformants were used in all analyses.

Real-time quantitative RT-PCR

Primer pairs for real-time PCR were designed using Primer Express (Perkin-Elmer, http://www.perkinelmer.com; Table S2). Gene-specific primers were chosen so that the PCR products were 80–130 bp. Total RNA (2 μg) was treated with Dnase I and used for first-strand cDNA synthesis using oligo(dT) primers and Taqman reverse transcription reagent (Applied Biosystems, http://www.appliedbiosystems.com/) at 25°C for 10 min and then 48°C for 30 min. For real-time PCR, the reaction mixture consisted of cDNA first-strand template, primer mix (5 μM) and SYBR Green Master Mix (Applied Biosystems) in a total volume of 25 μl. The PCR conditions were: 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The reactions were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). A relative quantification real-time PCR method was used to compare expression of the genes in mutant and overexpressing lines with WT (Panchuk et al., 2002). Relative quantification describes the change in expression of the target gene in a test sample relative to calibrator sample. Here *actin* was used as the internal control. The sample of WT was used as the calibrator, with the expression level of the sample set to 1. Each data point is the mean value from biological triplicate determinations.

Protein gel-blot analysis

Fourteen-day-old seedlings were treated at 37°C for 2 h, then 200–300 mg plant material were ground in liquid N_2, mixed with 600 μl buffer (10 mM HEPES/KOH, pH 7.9, containing 400 mM NaCl, 0.5 mM DTT, 0.1 mM EGTA, 5% glycerol and 0.5 mM PMSF), then clarified by centrifugation (12 000 **g** for 20 minutes). Samples corresponding to 30 mg of protein were resolved by SDS–PAGE, then electroblotted onto nitrocellulose membranes. The blots were incubated for 12 h at room temperature in PBS containing 5% w/v non-fat dry milk, and then incubated in PBST containing the appropriate antibody for 2 h at room temperature. Secondary anti-rabbit or anti-mouse antibodies (Sigma-Aldrich, http://www.sigmaaldrich.com/) conjugated to alkaline phosphatase were used for detection by enhanced chemiluminescence.

Gel mobility shift assay

Extraction of whole-cell extracts from 10-day-old *A. thaliana* seedlings was performed according to the method described by Li *et al.* (2004). Heat shock was applied by incubating the whole-cell extract in a water bath at 37°C for 30 min. The sequences and labeling of HSE oligonucleotides and the gel mobility shift assay were performed as described by Li *et al.* (2004).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. The FRET image as a control.

Figure S2. Fluorescence intensity and FRET images of *AtHSFA1a–CFP* and *AtCBK2–YFP*.

Figure S3. Purification of recombined AtCBK3–GST and AtHSFA1a–GST proteins by GST affinity chromatography.

Figure S4. Action of added CaM on the activity of AtCBK3.

Figure S5. Southern blot analysis of AtCBK3.

Table S1. Primer pairs used for amplifying coding regions of genes in the yeast two-hybrid assay.

Table S2. Primers used for quantitative real-time PCR.

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